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Practical
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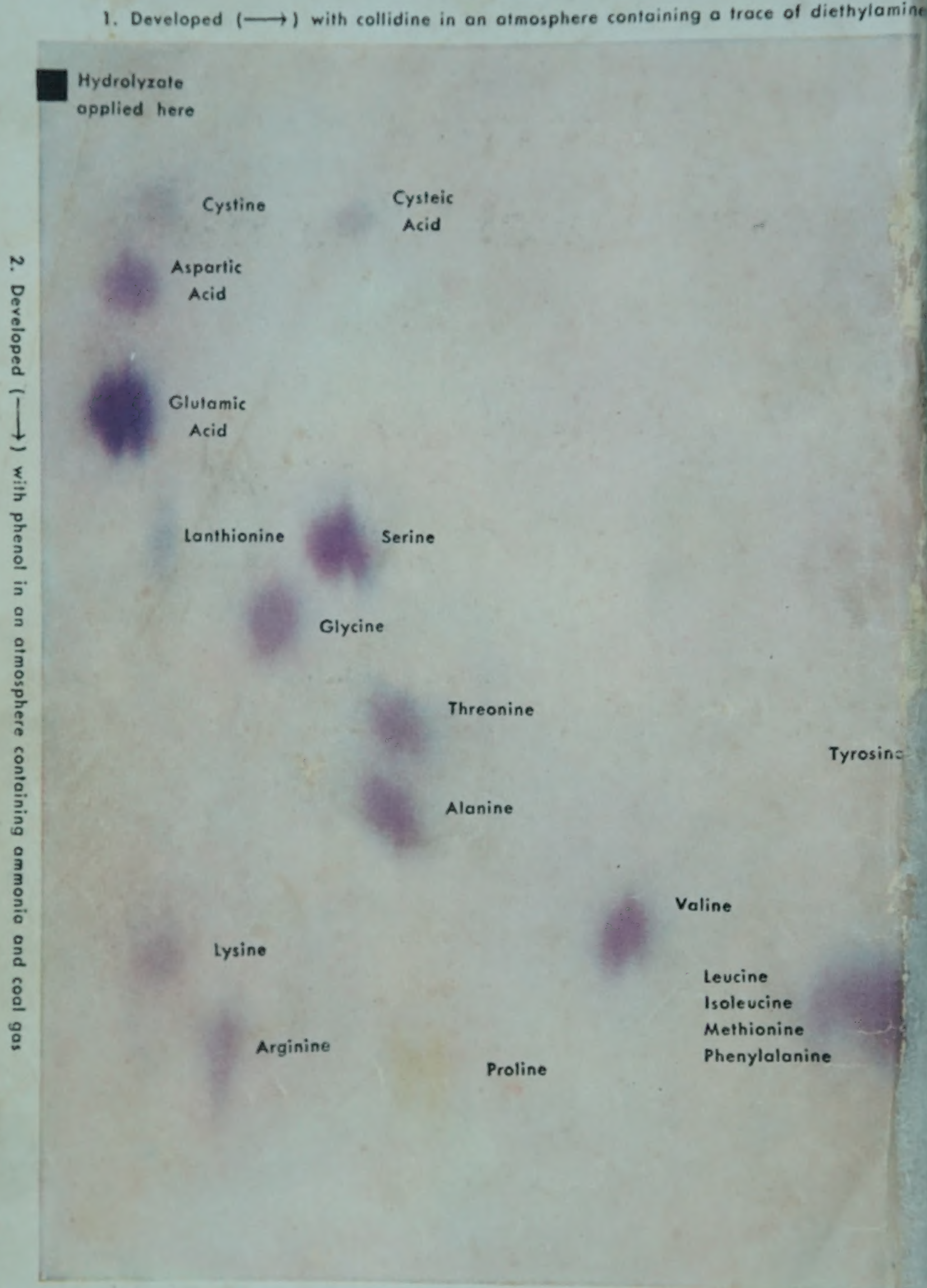
1. Developed (⟶) with collidine in an atmosphere containing a trace of diethylamin
Hydrolyzate applied here
Cystine
Cysteic Acid
Aspartic Acid
Glutamic Acid
Lanthionine
Serine
Glycine
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Tyrosin
Alanine
Valine
Lysine
Leucine
Isoleucine
Methionine
Phenylalanine
Arginine
Proline
2. Developed (⟶) with phenol in an atmosphere containing ammonia and coal gas

Two-dimensional chromatogram of tips of wool fibers. A drop of the hydrolyzate is placed
corner of the sheet of paper. This is hung from the long side and the chromatogram devel
s-collidine (1). The paper is dried, hung from the short side, and the chromatogram develo
phenol (2). It is dried again, sprayed with ninhydrin, and heated. Exposure to weather
has converted some cystine to cysteic acid and some to lanthionine. Otherwise the picture re
that of pure wool. The clump of unresolved amino acids in the right-hand bottom corner can
arated if benzyl alcohol and butanol are used in place of collidine and phenol, but then t
acids are crowded together. (Courtesy, ENDEAVOUR, The Imperial Chemical Industries, Ltd., Lon

Practical Physiological Chemistry

By

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Army Chemical Center, Maryland

Thirteenth Edition

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THIRTEENTH EDITION

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Library of Congress Catalog Card Number: 54-9278

PRINTED IN THE UNITED STATES OF AMERICA

BY THE MAPLE PRESS COMPANY, YORK, PA.

Preface to the First Edition

The plan followed in the presentation of the subject of this volume is rather different, so far as the author is aware, from that set forth in any similar volume. This plan, however, he feels to be a logical one and has followed it with satisfactory results during a period of three years in his own classes at the University of Pennsylvania. The main point in which the plan of the author differs from those previously proposed is in the treatment of the food stuffs and their digestion.

In Chapter IV the "Decomposition Products of Proteids" has been treated although it is impracticable to include the study of this topic in the ordinary course in practical physiological chemistry. For the specimens of the decomposition products, the crystalline forms of which are reproduced by original drawings or by microphotographs, the author is indebted to Dr. Thomas B. Osborne, of New Haven, Conn.

Because of the increasing importance attached to the examination of feces for purposes of diagnosis, the author has devoted a chapter to this subject. He feels that a careful study of this topic deserves to be included in the courses in practical physiological chemistry, of medical schools in particular. The subject of *solid tissues* (Chapters XIII, XIV and XV) has also been somewhat more fully treated than has generally been customary in books of this character.

The author is deeply indebted to Professor Lafayette B. Mendel, of Yale University, for his careful criticism of the manuscript and to Professor John Marshall, of the University of Pennsylvania, for his painstaking revision of the proof. He also wishes to express his gratitude to Dr. David L. Edsall for his criticism of the clinical portion of the volume; to Dr. Otto Folin for suggestions regarding several of his quantitative methods, and to Mr. John T. Thomson for assistance in proof reading.

For the micro-photographs of oxyhaemoglobin and haemin reproduced in Chapter XI the author is indebted to Professor E. T. Reichert, of the University of Pennsylvania, who, in collaboration with Professor A. P. Brown, of the University of Pennsylvania, is making a very extended investigation into the crystalline forms of biochemic substances. The micro-photograph of allantoïn was kindly furnished by Professor Mendel. The author is also indebted for suggestions and assistance received from the lectures and published writings of numerous authors and investigators.

The original drawings of the volume were made by Mr. Louis Schmidt whose eminently satisfactory efforts are highly appreciated by the author.

Philip B. Hawk

Philadelphia

Dedicated
To the memory of
LAFAYETTE B. MENDEL,
Guiding Genius of American Biochemistry
who fifty years ago inspired the
First Edition of this work

The Publishers are proud of the long record of continuous authorship of this book under the Blakiston imprint and are grateful for the professional acceptance which has made a Thirteenth Edition possible.

Preface

It is with a mingled sense of pride and humility that the senior author presents this—the Thirteenth Edition of PRACTICAL PHYSIOLOGICAL CHEMISTRY. To have been actively engaged in the authorship of successive editions of a textbook for fifty years is a privilege granted to few in the annals of medical publication. For this good fortune, I am deeply grateful.

Perhaps even more than some of its predecessors, this Edition has undergone extensive revision and expansion. The proportion of the book devoted to textual material, as distinguished from experiments and procedures, has increased to the extent that it now accounts for about one-half of the entire volume. Certain chapters, such as those dealing with Nucleic Acids and Nucleoproteins (Chapter 7), Milk (Chapter 8), Muscular Tissue (Chapter 10), Enzymes and Their Action (Chapter 12), and Isotopes (Chapter 32), have been exhaustively rewritten. To other chapters major additions have been made, as for example Chapter 18 to which a section on Liver Function has been added, and Chapter 23 which now contains a section on Microclinical Chemistry.

Try as its author will, no textbook in such a rapidly developing area of research as biochemistry, can possibly be kept strictly up to the minute; it must inevitably run a losing race with the periodical literature. Nevertheless the authors of this volume and their collaborators have expended every effort in the direction of supplementing established knowledge with current facts, theories, and hypotheses, insofar as this is possible, with full awareness that in some respects, obsolescence commences with the date of publication (if not before). This may be of more concern to the research worker than to the student and for that reason the former should have recourse to original journals such as those cited profusely throughout this book.

An endeavor has been made to keep pace with current advances in the many fields of biochemistry which are benefiting from the availability of advanced techniques and instrumentation. Symbolic of these innovations, which give promise of epoch-making advances, is the replacement of the time-honored Frontispiece of this book depicting absorption spectra of the blood pigments and their derivatives with a new color plate showing a two-dimensional chromatogram of a protein hydrolyzate. For this illustration the authors are greatly indebted to Dr. A. J. P. Martin of the National Institute for Medical Research, London, and to the publishers of ENDEAVOUR, The Imperial Chemical Industries, Ltd., London.

Experimental, analytical, and preparative methods employing these newer techniques and research tools are scattered through various chapters of the book. Only a brief summary can be given here. The student and teacher of biochemistry will find of general interest the new material on electrophoresis; the ultracentrifuge; ion-exchange resins; column and paper chromatography; countercurrent distribution; the helical structure of proteins; the polypeptide sequence of insulin; the chemistry of the corticosteroids; the synthesis of oxytocin; the chemistry and metabolism of nucleoproteins, nucleic acids and derivatives; the role of muscle proteins and ATP in muscle contraction; the biochemistry of bone and teeth; the kinetics of enzyme action; theories of blood clotting; the intermediary metabolism of carbohydrates, fats, and amino acids, including the role of coenzyme A and "one-carbon fragments"; the cobalamins, thioctic acid, leucovorin, and related factors; isotopes and their use in biochemical research; new material on antibiotics; and much more.

The teacher will find many new experiments suitable for class use, some of which were developed specifically for this Edition, illustrating such topics as paper and column chromatographic separation of amino acids, purine nucleotides, etc.; countercurrent distribution; myosin and actin glycogen storage and depletion; determination of blood volume wi isotope-labeled red blood cells; blood coagulation and prothrombin tim liver function tests; and many others.

The clinician will find new and authoritative sections on: the biochemistry of liver disease; the biochemistry of the bones and teeth, and the use of fluoride in the prevention of dental decay; isotopes and their use in medicine; dextran; cholinesterase; adrenal cortical and pituitary hormones; and the nutritive value of milk. Clinical chemical procedures not found in the previous Editions include the more important liver-function tests (thymol turbidity, cephalin-cholesterol flocculation, bromsulfalein, etc.); determination of blood protein-bound iodine, of blood cholinesterase, and of blood sodium and potassium by flame photometry; and others. A unique and valuable section has also been added on the use of micromethods in clinical chemistry, with detailed procedures and descriptions of apparatus.

Revisions of PRACTICAL PHYSIOLOGICAL CHEMISTRY have never been a one-man job. The success and prestige of the book has been due in no small measure to the fine cooperation shown by many teachers and investigators in the medical and biochemical fields. A list of those who have participated in some degree on one or more editions would read like a combination of WHO'S WHO and WHO WAS WHO in these professions.

In the elaboration of this Edition the authors feel signally honored to have enjoyed the cooperation of many distinguished scientists and educators. To credit them individually with specific contributions would fail to do justice to those whose advice and assistance is reflected in several places, if not generally, throughout the book.

In consequence, deep gratitude is expressed here on behalf of my associated co-authors and myself for major contributions and assistance of the collaborators whose names and affiliations are listed immediately preceding this Preface. Without the splendid and gracious help of these eminent biochemists and physicians it would have been infinitely more difficult to produce what we believe is the finest of a long series of revisions.

Special acknowledgment and thanks are due the following eminent experts and teachers whose generous assistance, both solicited and unsolicited, while not quite as extensive as that of the aforementioned colaborators was none the less welcome: Dr. Zoe E. Anderson, Director of he National Dairy Council's Research and Nutrition Service; Dr. A. K. Balls, Professor of Enzyme Chemistry, Purdue University; Dr. Albert L. Chaney, Director of the Albert L. Chaney Chemical Laboratory, Glendale, Cal.; Dr. L. C. Craig, Member, Rockefeller Institute for Medical Research; Dr. Alexander L. Dounce, Assistant Professor of Biochemistry, University of Rochester School of Medicine and Dentistry; Dr. Theodore E. Friedemann, Scientific Director, Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Col.; Dr. Linus Pauling, Professor of Chemistry, California Institute of Technology; Dr. Kurt G. Stern, Adjunct Professor of Biochemistry, Polytechnic Institute of Brooklyn; Dr. Henry Tauber, Associate Professor of Experimental Medicine, School of Public Health, University of North Carolina; Dr. Oscar Touster, Associate Professor of Biochemistry, Vanderbilt University School of Medicine; Dr. Everett C. Cogbill and Mr. Richard M. Rush of the University of Virginia; and Dr. M. A. Derow of Boston University.

Once again it is my pleasure to pay tribute to the skill and untiring efforts of my associates, Dr. Bernard L. Oser, whose more than quarter-century association with this book is well known to its friends, and Dr. William H. Summerson, whose long experience as a teacher and investigator at Cornell Medical College preceding our association, eminently qualified him for the important role he has assumed as a co-author.

For their assistance in matters editorial the authors are particularly indebted to Mrs. Eunice Stevens, editor-in-chief of Blakiston, and her able and conscientious associate editor, Mr. Barney Pisha. Their efforts in standardizing style and typography are reflected in the enhanced appearance of this completely reset edition. In this connection reference may be made to questions of spelling and nomenclature where uniformity of practice in the scientific literature has yet to be achieved. Since the American Chemical Society, more than any other single organization, has been concerned with standardization in this field, the recommendations of that organization have been adopted despite the risk, in some instances, of jarring the sensibilities of a few readers (and occasionally of a co-author). While appreciation is expressed to the publisher's and printer's staffs for their careful efforts in proofreading, the authors assume full responsibility for such errors and oversights as will inevitably be discovered by sharp-eyed readers.

Appropriate acknowledgment has been made through this edition to the numerous authors, publishers and instrument and apparatus companies who so graciously granted permission for the use of illustrations, tables, or other copyrighted material. Appreciation is expressed here for these courtesies and, in the unlikely event that acknowledgment has been omitted in the specific places where such material has been used, the authors claim human frailty and apologize for these oversights.

Finally, the authors are deeply appreciative of the patience and cooperation of the Maple Press whose fine craftsmanship has made possible the production of this attractive volume.

I trust I will be pardoned for concluding this Preface with a short personal note.

The manuscript of the First Edition of this book was drafted high up in the old Hare Laboratory of the Medical School of the University of Pennsylvania, amid the stimulating fragrance of the Department of Anatomy. It served as the basis for a course of instruction even before the edition was in print. Now, a half-century later, and thanks to its friendly reception by the profession, the Thirteenth Edition has rolled off the press.

Among those to whom I was indebted for its genesis, first place must go to the late Professor Lafayette B. Mendel for it was he who generated the spark of inspiration. The value of Dr. Mendel's suggestions and criticism of the manuscript of several editions was great indeed. His wonderful letters, written in long hand with purple ink and crammed full of ideas, references, and suggestions were of incalculable aid. These missives were never less than six pages long and often twice as long. The First Edition was thus, quite naturally, based on the courses as given at Yale University, with minor features as adopted at Columbia University (College of Physicians and Surgeons) where I passed my first teaching assignment (1901–1903) under Professor William J. Gies. Dr. Mendel's interest in the book never flagged. It is therefore fitting and proper that to Dr. Lafayette B. Mendel, guide, philosopher, and friend of many an outstanding biochemist as he was of the senior author, should be dedicated this "Golden Anniversary" Edition of PRACTICAL PHYSIOLOGICAL CHEMISTRY.

PHILIP B. HAWK

April 1954
MIAMI BEACH, FLORIDA

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1

Physicochemical Principles

Living matter differs from nonliving in its possession of certain characteristic properties such as growth, reproduction, respiration, and motion. The science of physiological chemistry deals with the application of chemical and physicochemical principles and methods to the study of these phenomena. In the early days of the science this meant the analysis of foods entering the organism and of excreta leaving it; it involved the study of the composition of the various tissues and organs of the body, the blood, the digestive secretions, etc. In this way a great deal of information has been collected concerning the composition of living matter and the fate of the substances that are necessary for continuance of life and growth. Although far from complete these researches have progressed to the point where we now possess a fairly comprehensive picture of the gross changes that take place in protoplasm.

The experimental methods used, however, for the most part involved the destruction of the living cell. In recent years the emphasis has been placed on the mechanisms concerned in the reactions of the living protoplasm itself. Since protoplasm is largely water, this requires a study of the nature of solutions and the complex behavior of mixtures of electrolytes. Since the physical basis of protoplasm is colloidal in character, a study of the peculiar structure and properties of colloidal solutions is also necessary. Some of the more important physicochemical principles that are finding wide and fruitful application in the study of life phenomena are discussed briefly in the following pages.

THE COLLOIDAL STATE

True and Colloidal Solutions. Thomas Graham in 1861 classified all substances into two groups, crystalloids and colloids, depending upon their ability to diffuse through membranes such as parchment. According to Graham, crystalloids readily passed through parchment membranes while colloids did not. We now recognize that matter cannot be classified in this manner since many typical colloids, such as certain proteins, are crystallizable, and practically all crystalloids may, under proper conditions, be brought into the colloidal state.

According to modern concepts, colloidal solutions, instead of being solutions of particular types of matter, are solutions with a characteristic kind of structure. Substances such as glucose or sodium chloride, which form true solutions in water, disintegrate, when dissolved, into individual molecules or ions which are less than 1 mμ (1 millionth mm.) in diameter.

The smallest particle that can be seen with a high-power light microscop has a diameter of about 200 mμ. By means of an electron microscope, part cles with a diameter as small as 10 mμ are readily made visible. When th particles of solute are larger than 200 mμ they are said to be in suspension on standing such particles will gradually separate out. When, however the solute is dispersed into particles which are intermediate in size be tween ordinary molecules such as exist in true solutions and the coars particles found in suspensions, it is said to be in the colloidal state, an solutions containing particles of that size are known as *colloidal solution* or *sols*. Sols which have become jellylike are called *gels*.

Colloidal solutions, true solutions, and suspensions thus differ fron each other fundamentally only in the size of the particles of *solute* (th *disperse phase*) dispersed in the *solvent* (the *dispersion medium*). Becaus of the dimensions of the disperse phase, colloidal solutions exhibit certai characteristic and unique properties (to be discussed in later sections which confer upon them their great importance in the structure of living protoplasm. This importance resides in the fact that protoplasm is considered to be a complex system containing many different crystalloida and colloidal components. Although the structure and properties of this system are too complex to permit exact characterization in the present state of our knowledge, we may gain an insight into these questions by a study of similar, though very much simpler, colloidal systems such as are discussed below.

Preparation of Colloidal Solutions. The relationship between colloidal solutions, true solutions, and suspensions indicated above suggests two general methods by which colloidal solutions may be prepared. These methods are classified as (1) condensation and (2) dispersion methods, depending upon whether the colloidal particles are formed by aggregation individual molecules or by disintegration of coarse particles of matter

CONDENSATION METHODS. The principles underlying the preparati of colloidal solutions by condensation methods are similar to those i volved in ordinary precipitation reactions. In both processes the solu is permitted to become supersaturated with respect to some partic substance. Such supersaturated solutions, in the presence of suitable condensation nuclei, develop molecular aggregates which continue to increase in size as long as any available material remains in solution. In precipitation reactions this process of growth continues until the particles become visible in a microscope or to the naked eye when they flocculate from solution. By proper regulation of the experimental conditions, which differ for different substances and procedures, the growth of molecula aggregates may be checked when the particles attain the size charac teristic of the colloidal state, thus forming colloidal solutions. Whether particular reaction will lead to the formation of a colloidal solution or visible precipitate depends, therefore, entirely upon the conditions unde which the experiment is carried out. Von Weimarn, who studied thi question very extensively, showed that by merely varying the concentra tions of $Ba(CNS)_2$ and $MnSO_4$ from N/20,000 to 7 N, the form of th $BaSO_4$ precipitated could be made to vary from large crystals to a colloida gel. The colloidal state, as indicated in the following scheme, is merely a ntermediate stage between coarse precipitates and true solutions which nay be approached, under proper conditions, from either direction.

Condensation Methods→

True Solutions	*Colloidal Solutions*	*Suspensions*
Molecules and Ions	Molecular Aggregates	Molecular Aggregates
Diameter: Less than 1 mμ	Diameter: 1 mμ to 200 mμ	Diameter: Greater than 200 mμ

←Dispersion Methods

ost of the inorganic colloids may be formed by condensation methods volving such reactions as reduction, oxidation, hydrolysis, and double composition. Thus if a dilute solution of gold chloride is treated with rmaldehyde under proper conditions, the gold ions are reduced to atoms gold which then aggregate into particles of colloidal size. Practically all f the metals yield colloidal solutions under similar conditions. Boiling a very dilute solution of ferric chloride results in hydrolysis of this salt with he formation of a colloidal solution of ferric hydroxide. Similar solutions obtained by using salts of Cr, Al, or Sn. A dilute solution of ide in water, when treated with hydrogen sulfide, undergoes decomposition with the formation of colloidal arsenious sulfide. In reparation of colloidal solutions by these methods it is essential that actions used do not lead to the formation of soluble, strong electro- This condition is important because colloidal solutions of the type ussed here are extremely sensitive to small amounts of electrolytes, ch cause aggregation of the colloidal particles into larger particles ch precipitate out of solution.

ISPERSION METHODS. The dispersion methods for preparing colloidal tions involve, as indicated above, the subdivision of coarse material particles of colloidal size under conditions which will prevent coales- e of those particles. Many substances may be reduced to approxi- ely colloidal size by grinding in a colloid mill, which consists essen- ly of two flat metal plates placed almost in contact and rotated at very n speeds in opposite directions. Colloidal solutions of most of the metals y be prepared by producing an electric arc between electrodes of the al held under water or some other suitable liquid. It is quite probable thod involves some condensation of metal vapors as well as he metal itself. Solutions prepared by either of these dually flocculate unless some stabilizing agents are added to prevent coalescence of the particles. For this purpose use is made of certain substances, either electrolytes or other colloids, called peptizing agents. The electrolytes usually act by conferring an electrical charge on the colloidal particle, which is an essential condition for stability of colloidal solutions of this class. Peptizing colloids, also called protective colloids, apparently form a film about the individual particles, which into particles resembling the protective agent in stability and other properties.

General Properties of Colloidal Solutions. It has already been pointed out that the essential difference between colloidal and true solutions lies in the size of the particles of solute dispersed in the solvent.

Although the particles in a colloidal solution are too small to be retain by ordinary filter paper, they are too large to pass through such me branes as collodion, parchment, or cellophane, which are permeable most substances in true solution. This inability of the colloidal partic to diffuse through certain membranes is made use of in the process kno as *dialysis*, by which colloidal solutions may be freed from noncolloi impurities. The solution is placed in a suitable dialyzing bag and is s pended in a large volume of distilled water which is changed at frequ intervals until the liquid inside the bag no longer gives a test for the particular substance to be removed. This process of dialysis is used ex sively in the preparation of salt-free solutions of such biological col as the proteins. Last traces of electrolytes may be removed by the p known as *electrodialysis*, in which an electrical current is passed th the solution in a suitably designed apparatus.

If a solution containing colloidal particles is forced through a suit membrane by pressure, the membrane becomes an ultrafilter, and colloidal particles are separated from the solution. This process is ca *ultrafiltration*. By this means it is possible to separate the crystalloid and colloidal components of such a fluid as blood plasma, for exampl Furthermore it is possible to prepare membranes containing pores almost any desired size, so that colloidal particles of any particular may be separated from smaller particles as well as from material in solution.

Another widely used method for separating colloidal particles of dif ent sizes is based upon the use of the high-speed centrifuge or *ultra trifuge*. This apparatus, originally developed by Svedberg for the stud particle size in suspensions of inorganic colloids, has found extende plication to the problems of protein and virus chemistry by Svedbe many others, until today it is a fundamental research instrument i fields. With this instrument a sedimenting force up to several hu thousand times the force of gravity can be applied to particles in co suspension. Naturally the heavier particles will settle out of the sol under these conditions faster than the lighter or smaller particles the actual separation and isolation of individual colloidal substanc been achieved in this way. Furthermore by the use of suitable o devices it is possible to follow the rate of settling of the various ty colloidal particles which may be present, and thus estab[illegible] whether the disperse phase consists of colloidal particles of [illegible] ent sizes (*heterogeneous*) or of only one size (*homogeneous*), but also the average size of the particles themselves. If it can be shown that the colloidal particle is a single molecule of the substance, the particle size the becomes a measure of the molecular weight of the substance. This metho of approach has found particular application in the field of protei chemistry.

The sedimentation of a particle under stated conditions may be characterized by the *sedimentation constant*, s, defined as

$$s = \frac{dx/dt}{x\omega^2}$$

where dx/dt is the rate of settling of the particle per unit time at a distance x from the center of rotation of the centrifuge, and ω is the angular velocity in radians per second. Since the numerical value of s is usually very low—around 1×10^{-13} for proteins—sedimentation constants are commonly expressed in terms of a unit, the *Svedberg, S*, which equals s multiplied by 1×10^{13}.

The relation between the sedimentation constant and the molecular weight under ideal conditions is given by the following expression:

$$M = s \cdot \frac{RT}{D(1 - \bar{v}d_m)}$$

where M is molecular weight, R is the gas constant, T the absolute temperature, D the diffusion constant, $\bar{v}$ the partial specific volume of the substance (the volume occupied by one gram of substance at infinite dilution in the solvent) and d_m the solvent density. This equation requires separate determination of the diffusion constant D; errors in D will be reflected in the value for the molecular weight. Nevertheless the equation, suitably corrected for deviations from ideal conditions, has been widely used. Examples of the molecular weights of proteins determined by sedimentation velocity will be found in Chapter 5.

An alternative procedure for the determination of particle size or molecular weight in the ultracentrifuge is based upon the attainment of a *sedimentation equilibrium*. In this the rate of settling of the particles is established at such a point that it exactly equals the opposing force of diffusion, which tends to redistribute the solute throughout the solvent. Under these conditions the molecular weight is obtained by means of the equation:

$$M = \frac{2RT \ln \dfrac{C_2}{C_1}}{\omega^2(1 - \bar{v}d_m)(x_2^2 - x_1^2)}$$

where C_1 and C_2 are the concentrations at distances x_1 and x_2, respectively, from the center of rotation. (The symbol "ln" stands for the natural logarithm.) The value of the diffusion constant is not required in this method, and this is an advantage. On the other hand, precise establishment of sedimentation equilibrium requires careful control over sedimentation rate for relatively long periods of time. In general, molecular weights determined by sedimentation equilibrium differ but slightly from those obtained by sedimentation velocity.

By noting and measuring deviations from the equations just presented, which are based upon spherical particles obeying the gas laws, information concerning not only the size but also the shape of particles may be obtained. In the case of proteins, variation in the value of the sedimentation constant with concentration has been interpreted in terms of equilibrium between monomeric and dimeric forms of the protein molecule.

Since the particles in a colloidal solution consist either of very large olecules or of aggregates of large numbers of individual molecules, it llows that a colloidal solution will contain only a minute fraction of the number of particles present in a true solution of the same concentration. Such physicochemical properties of solutions as vapor pressure and osmotic pressure depend almost entirely upon the number of particles in solution, the chemical nature of these particles being immaterial. Colloidal solutions, with their comparatively small number of particles, therefore exhibit only very small osmotic pressures; and their vapor pressures, as well as related boiling and freezing points, are practically the same as those of the pure dispersion medium.

If a concentrated beam of light is passed through a colloidal solution and viewed at right angles, its path through the solution is plainly visible as a milky turbidity. True solutions under similar conditions appear optically void. This phenomenon, known as the Tyndall effect, is due to the reflection and scattering of the light by the particles in solution and is similar to that observed when a beam of sunlight passes through the dust-laden atmosphere of a darkened room.

Siedentopf and Zsigmondy, in 1903, developed the *ultramicroscope*, in which a very fine, intense beam of light is focused in a colloidal solution and its path observed with a microscope placed at right angles to the beam. The true nature of the Tyndall effect becomes apparent when colloidal solutions are viewed with the ultramicroscope. It is then observed that the Tyndall light is composed of individual points of light, each point representing light reflected by an individual particle. Colloidal solutions are thus shown to consist of discrete particles suspended in the dispersion medium. When viewed through the ultramicroscope the particles in a colloidal solution are seen to be in a continual state of violent vibratory motion. This phenomenon is known as the *Brownian movement* and is due to bombardment of the particles of disperse phase by the molecules of the solvent.

Colloidal particles ordinarily carry electrical charges which are considered to be distributed over the surface of the entire particle. Because of the presence of these charges the particles in a colloidal solution migrate toward one of the poles in an electrical field, the process being known as *cataphoresis* or, more commonly, *electrophoresis*.

The distance x through which a particular colloidal particle moves under the influence of an electric current is proportional to the time t and to the potential gradient E at the spot where the particle is. E in turn depends upon the current i, the conductivity of the solution λ, and the cross-sectional area q of the solution at this point. The proportionality constant is known as the electrophoretic mobility μ. Hence $\mu = x/tE = x/t \times q\lambda/i$. The dimensions of μ are therefore square centimeters per volt per second. The distance x is usually measured by the so-called moving boundary method. In the version of this procedure developed by Tiselius and widely used, the colloidal solution is brought into contact with the solvent alone by a process of layering that uses a sectioned U-tube (see Fig. 105, p. 461) to produce a sharp boundary between the colloid-containing solution and the solvent alone, although the two solutions are continuous through the solvent itself. An electric current passing across this boundary will carry the colloidal particles into the solvent or away from it, depending upon the polarity of the current and the charge on the particles. If all the particles are similar with respect to charge, mass, and shape, they will move as a unit, so that in effect the boundary itself will move and will remain sharp. Optical or other methods for measuring the movement of the boundary will then establish the rate of movement of the colloidal material. If the colloidal solution is composed of various types of particles with differing electrophoretic mobilities, several boundaries or zones will develop, moving at different rates. In this way a mixture of colloidal substances may be characterized, and under suitable conditions the components may actually be separated from one another.

A simplified form of electrophoresis apparatus suitable for clinical and other uses employs a strip of filter paper (*paper electrophoresis*) saturated with solvent (electrolyte) and attached to two electrodes. The colloidal solution is deposited on the filter paper at one spot, the filter-paper matrix tending to localize the added material. Under the influence of an electric current the colloidal particles present will migrate along the paper strip in bands in much the same manner as that described above for the Tiselius procedure, and these bands may be visualized by suitable procedures (see Fig. 57, p. 184).

Like the ultracentrifuge mentioned above, electrophoresis in the study of colloidal systems has found application not only in isolating individual colloidal substances from mixtures but also in characterizing the components of complex natural colloidal solutions such as the proteins of blood plasma.

Classification of Colloidal Solutions. Colloidal solutions are classified into (1) suspensoids, or lyophobic colloids, and (2) emulsoids, or lyophilic colloids. The *suspensoids* include such colloidal solutions as those formed by the metals, inorganic salts, etc., and their preparation usually requires special methods such as the dispersion and condensation methods described above. They are called *lyophobic* colloids because there is no affinity between the particles of disperse phase and the dispersion medium. In many of their physical properties, such as viscosity, suspensoids differ only slightly from the pure dispersion medium. The particles of a suspensoid carry a definite electrical charge which may be changed only by very special methods. Suspensoids are flocculated by very small quantities of electrolytes, and when so precipitated cannot ordinarily be brought back into colloidal solution. The precipitation of suspensoids is therefore irreversible. When viewed with the ultramicroscope suspensoids are seen to contain well-differentiated particles in vigorous Brownian movement.

The *emulsoids*, since they include the proteins and the higher carbohydrates, are of much greater biological importance and interest than the suspensoids. They are called *lyophilic* (or *hydrophilic* when the dispersion medium is water) colloids because they have a high affinity for the dispersion medium. The viscosity of emulsoids is usually much higher than that of the dispersion medium. The particles carry electrical charges which, in the case of proteins, for example, may be changed in sign or magnitude by such simple measures as changing the acidity or alkalinity of the solution. The emulsoids require large amounts of electrolytes for their precipitation and when precipitated may usually be brought back into colloidal solution by the addition of fresh solvent. The precipitation of emulsoids is therefore reversible.

Emulsoids and suspensoids thus exhibit essential differences in their behavior toward the solvent and in their sensitivity to small amounts of electrolytes. The stability of suspensoids depends entirely upon the charges on the particles, which permit them to repel each other and thus prevent aggregation into coarser particles. If the charges on the suspensoid particles are neutralized, or reduced below a certain critical value, the particles coalesce and precipitate. The sensitivity of suspensoids to small amounts of electrolytes is therefore due to the fact that electrolytes neutralize or reduce the charges on the particles and thereby remove the force which is ordinarily sufficient to prevent aggregation and flocculation. In this precipitating action of electrolytes on suspensoids, the active ion is the one whose charge is opposite in sign to that carried by the suspensoid.

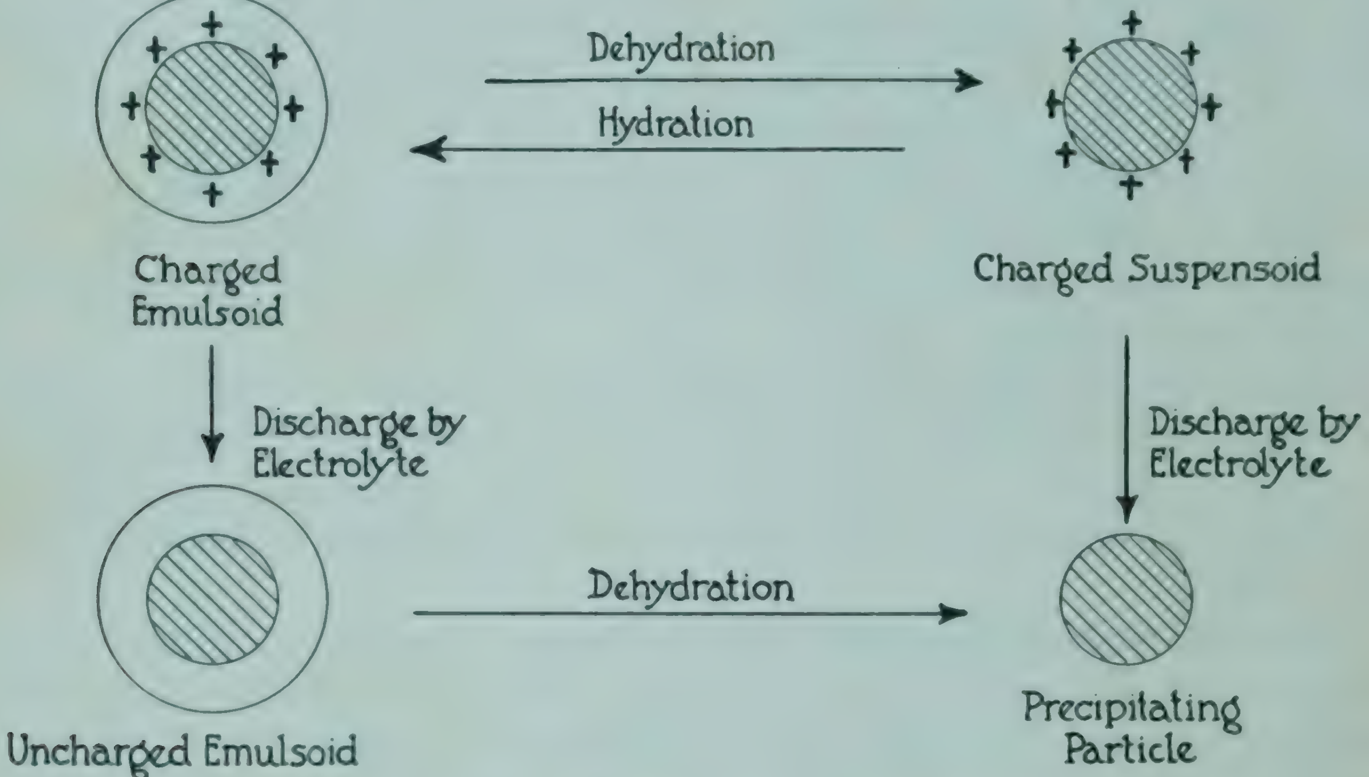

FIG. 1. STABILITY OF SUSPENSOIDS AND EMULSOIDS.

On the other hand, emulsoids possess two stability factors—charge and hydration—either of which is capable of preventing the aggregation and flocculation of the colloid. Neutralization of the charges on the emulsoid converts it into a neutral, or isoelectric, colloid which is perfectly stable as long as the particles remain hydrated. Dehydration of a charged, electrolyte-free emulsoid (e.g., by the addition of a dehydrating agent like alcohol) converts it into a suspensoid exhibiting the characteristic sensitivity toward electrolytes. The precipitating effect of large amounts of such salts as ammonium sulfate or sodium chloride on emulsoids is due to the fact that saturated solutions of these salts act also as dehydrating agents, thereby discharging and dehydrating the particles at the same time. Fig. 1, from Kruyt, illustrates these differences in stability between emulsoids and suspensoids.

Under changing conditions of temperature and concentrations of disperse phase, hydrogen ions, and electrolytes, emulsoids have the property of imbibing large quantities of water and setting into semirigid gels. These gels are thought to possess a definite structure, consisting in some cases of a network of disperse phase, or hydrated material, enclosing some of the dispersion medium. On standing for a time most gels gradually contract, extruding a portion of the dispersion medium. This process is known as *syneresis*. The properties of imbibition and gel formation exhibited by emulsoids probably are responsible for the characteristic physical form of protoplasm. The swelling and shrinking exhibited by protoplasm are due probably to the hydration and dehydration of its colloids, presumably governed by the same laws that operate in the case of the simpler systems.

Colloidal particles possess appreciable surfaces which permit such phenomena as surface tension (*q.v.*) to come into play at the interfaces between the particles and the dispersion medium. The magnitude of these surfaces becomes evident when we reflect that if a 1-cm. cube is subdivided into cubes with 10-mμ edges, the surface area is increased from 6 sq. cm. to 600 sq. m. Substances such as most emulsoid colloids (soaps, proteins, etc.) which decrease surface tension tend to accumulate at surfaces, whereas other substances such as sodium chloride which increase surface tension tend to be less concentrated there. This increase in the concentration of a substance at a surface is known as *adsorption*. In the case of colloidal solutions, adsorption thus involves an increase in concentration in the boundary layer between dispersed phase and dispersion medium. The mechanism of adsorption is obscure; it has been suggested that the adsorbent enters into a loose combination with the material adsorbed by means of latent valences of atoms in the surface layers. In many cases (such as adsorption of many different kinds of gases, liquids, and even solids by such suspensions as charcoal), it is difficult to understand how any kind of chemical combination takes place. When electrolytes are adsorbed by the charged particles in colloidal solutions it is the ion with opposite charge which is adsorbed.

Adsorption probably plays a very important role in many of the reactions taking place in living protoplasm, since in this manner substances ordinarily present in low concentration may have their effective concentrations increased tremendously by being accumulated at boundary surfaces. Concentration by adsorption also finds use in the laboratory in the isolation from very dilute solution of such natural products as enzymes, vitamins, hormones, etc. The adsorbed material may be released (*eluted*) from the surface of the solid adsorbent (e.g., charcoal, metallic hydroxides, fuller's earth) by changing the acidity or surface tension of the solvent. In the process of chromatographic adsorption the solid adsorbent is packed into a tall glass column down which the solution containing adsorbable material is allowed to flow slowly. This procedure is discussed in detail in the section on chromatography (see p. 14).

The studies of Harkins and of Langmuir indicate that substances accumulate at boundary surfaces in a definite pattern. Thus molecules having both polar and nonpolar groups orient themselves at oil-water interfaces in such a fashion that the polar groups dissolve in the water while the nonpolar groups dissolve in the oil. Oleic acid, for example, orients itself at oil-water interfaces so that the polar carboxyl group is directed into the water, which it more closely resembles in general structure than does the nonpolar alkyl group, which is directed into the oil. Since protoplasm contains both water and lipide materials it is quite probable that some of the cell constituents are oriented in a similar fashion within the cell. It is also probable that the internal structure of the cell is based upon orientation of various types of molecules within the cell.

EXPERIMENTS ON COLLOIDAL SOLUTIONS

A. METHODS FOR PREPARATION OF COLLOIDAL SOLUTIONS

1. ***Preparation of Colloidal Solutions of Prussian Blue and of Arsenious Sulfide by Double Decomposition.*** **To 10 ml. of 0.02 N potassium ferrocyanide in a beaker add with mixing 10 ml. of 0.02 N ferric chloride. Dilute a portion of the mixture and note that there is no precipitate. This colloid is negatively charged.**

Mix 50 ml. of 1 per cent arsenious oxide solution (made by boiling the oxide with water) with 50 ml. of saturated solution of hydrogen sulfide. Heat to boiling (in the hood), filter and cool. This colloid is electronegative also.

2. ***Preparation of Colloidal Ferric Hydroxide by Hydrolysis.*** **To 200 ml. of boiling water add 1 ml. of 33 per cent ferric chloride solution, or about 0.3 g. of solid ferric chloride. Note the beautiful reddish-brown color. This is a positively charged colloid.**

3. ***Preparation of a Gold Sol by Reduction.*** **To 100 ml. of pure redistilled water add 1 ml. of 1 per cent gold chloride solution and 5 ml. of 0.5 per cent potassium carbonate solution. Heat to boiling, remove from the flame, and add two drops of 20 per cent formaldehyde solution. If necessary, heat again and add one more drop. Observe the changes in color as the particles of metallic gold become larger and larger. Most metallic colloids are electronegative.**

4. ***Preparation of Colloidal Platinum by Electrical Method.*** **Connect two short platinum wires (1 to 3 mm. cross-section) with stout copper wires and pass through pieces of glass tubing to serve as handles. Connect both wires with the terminals of a 110-V. d.c. lighting circuit with a lamp bank of five 100-watt lamps in parallel (to cut down the current to about 5 amperes). Place pure distilled water in a crystallizing dish about 4 inches in diameter, add a very slight trace of hydrochloric acid, and immerse the two platinum tips. Bring the tips together and then separate them by about 1 to 2 mm. An arc develops. Maintain this for several minutes. Filter.**

5. ***Preparation of Emulsoid Solution.*** **Prepare solutions of gelatin (5 per cent), agar (1 per cent), starch (2 per cent), and soap (2 per cent) by allowing the dry colloid to become thoroughly soaked in the required amount of water in a beaker at room temperature, followed by immersion of the beaker in a boiling water bath, stirring until solution is complete. Cool the solutions and note that gels are formed. Heat again in boiling water and note that the gels liquefy. On standing for some time the gels should show syneresis.**

B. GENERAL PROPERTIES OF COLLOIDAL SOLUTIONS

1. ***Diffusion Through Membranes (Dialysis).*** **Prepare a dialyzing bag from seamless cellulose dialyzer tubing[1] as follows: Cut a strip about 15 cm. long**

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[1] Obtainable from most laboratory supply houses in the form of rolls of varying length and width. If not available, collodion bags may be used. Pour 10 to 20 ml. of collodion solution

from the roll of flat tubing, wet the strip thoroughly with water, and separate the sides to form a tube. (This operation is facilitated by holding the strip vertically in a stream of running water.) Tie one end of the tube tightly with string to form a bag. Fill the bag with colloidal Prussian blue or ferric hydroxide solution (prepared as described above), tie loosely at the top with string, and suspend in a beaker of water. Observe at intervals. Do you detect any diffusion of the colloid through the membrane? Test the water in the beaker for chloride. Is any substance diffusing through the membrane?

2. *Diffusion Through Gels.* Place 5 ml. of 5 per cent gelatin in each of two test tubes. Cool until the gelatin solidifies. Pour copper sulfate over the gel in one tube and Prussian blue solution over the other. Let stand. Note the degree of diffusion of the blue color in the two cases. The experiment may be repeated with eosin and methylene blue, the former a noncolloidal and the latter a colloidal dye.

3. *Mutual Precipitation of Colloids.* Mix equal volumes of solution of the negative colloid arsenious sulfide and the positive colloid ferric hydroxide. Explain. Can you write the equation for any reaction which occurred?

4. *Precipitation of Colloids by Salts.* To a solution of Prussian blue, ferric hydroxide, or other suspensoid, add a few ml. of 10 per cent sodium chloride solution, and allow to stand. Treat a solution of an emulsoid such as gelatin or starch in the same way. Which are precipitated by the electrolyte? Add an excess of water to the precipitate. Is the colloid reversible? If the emulsoid is not precipitated saturate the solution with solid magnesium sulfate. To some of the precipitate add an excess of water and boil. Is the emulsoid reversible?

5. *Demonstration of a Protective Colloid.* To a mixture of three drops of concentrated nitric acid and 5 ml. of 0.05 N silver nitrate add 5 ml. of 0.05 N sodium chloride. Note the curdy precipitate. Repeat, but add 1 ml. of gelatin solution to the silver nitrate and to the sodium chloride solutions before mixing.

6. *Demonstration of Optical Properties of Colloids.* Pass a ray of brilliant light through solutions of typical colloids in beakers. (Colloidal solutions of gold, mastic, ferric hydroxide, or arsenious sulfide give good results.) Note the turbidities where the light strikes the solutions and compare with pure water and ferric chloride solutions, which should contain only traces of dust and other particles. Direct sunlight or arc lamp may be used. The light should be concentrated by means of a lens so that only a part of the solution is illuminated.

If the cone of light be viewed through a Nicol prism and the prism rotated, the field will become alternately light and dark, indicating polarization. Solutions of eosin or quinine fluoresce in the light, but the light is not polarized since these solutions are not colloidal. Also examine a colloidal gold solution under the ultramicroscope and note the Brownian movement of the particles.

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into a clean dry Erlenmeyer flask or large test tube. Rotate to form a complete film over the inner surface and clamp in an inverted position. After ten minutes loosen the edges of the film with a knife and run water from the faucet between the membrane and the container. Preserve under water until needed.

7. Viscosity (Internal Friction) of Colloidal Solution. Fill a 10-ml. pipet with water, suspend it in a vertical position, and allow it to drain. Count the number of seconds required for emptying. Repeat, using a solution of Prussian blue or ferric hydroxide. Repeat again, using a 2 per cent solution of gelatin. How does the viscosity of the suspensoid compare with that of the emulsoid and with that of pure water? A more accurate form of this apparatus is a viscosimeter of the Ostwald type (see Exp. 8). A solution of gelatin shows its lowest viscosity (most rapid emptying of the viscosimeter) at its isoelectric point (about pH 4.7).

8. Estimation of Viscosity with Ostwald Viscosimeter. The apparatus illustrated in Fig. 2 is used and is kept in a constant-temperature water bath during the determination. Test with water first. By means of a pipet introduce a suitable exact amount (usually 5 ml.) of water into bulb E. Blow at F and force the liquid above mark C. The fluid column should still reach to bulb E. Allow it to flow back and note the time with a stop watch for the meniscus to pass from C to D. Repeat until constant values are obtained. Repeat with gelatin or some other colloidal solution. If water requires 60 seconds and gelatin solution 120 seconds, the relative viscosity of the gelatin is 2.0.

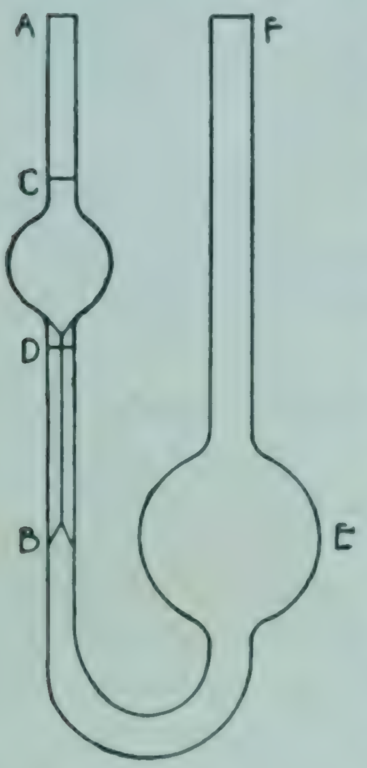

FIG. 2. OSTWALD VISCOSIMETER.

9. Adsorption by Charcoal. Caramelize a small amount of sugar by heating it in a crucible. Dissolve in 100 ml. of water, add 5 g. of animal charcoal, and boil for five minutes. Filter. The filtrate should be colorless. Charcoal is used for clarification in the commercial refining of sugar and in many similar processes. Repeat the experiment, using a dilute solution of crystal violet or other dye instead of the caramelized sugar solution. After the filter has drained, pour alcohol or acetone over the moist charcoal. Does the dye enter into a firm combination with the charcoal? Acetone and alcohol are more strongly adsorbed than crystal violet and tend to displace it from the charcoal. What is this displacement process called?

10. Electrophoresis of Colloids. Clamp in an upright position a glass U-tube (one with limbs about 15 cm. long and 2 cm. in diameter is satisfactory). Half fill the tube with a suitable colored colloidal solution (arsenious sulfide, ferric hydroxide). With a pipet slowly and carefully add distilled water to each limb to within about 2 cm. of the top. With suitable care a sharp boundary between the water and the colloidal solution is obtained. Place a platinum electrode in contact with the water in each limb, and connect each electrode to a terminal of known polarity from a source of 110 V. d.c. Note (1) the level of the colloid-water boundary in each limb and (2) the polarity of the electrodes, and turn on the current. Allow it to run until definite movement of the boundary has taken place. What does this experiment prove with regard to the sign of charge on the colloidal particles? Repeat with a colloid of opposite charge.

DONNAN EQUILIBRIUM

The British physicist Donnan has made a contribution of the utmost importance in physiology to our knowledge of the influence of ions in colloidal systems. Let us suppose a membrane (indicated below by a vertical line) to be impermeable to the anion R of a salt NaR in solution on one side of the membrane but permeable to all other ions and salts involved. Let us further suppose that we have at the beginning of the experiment NaR on one side of the membrane and NaCl on the other. (See diagram at left, below.) NaCl will diffuse from 2 to 1 until at equilibrium conditions will be as indicated on the right.

Na^+	Na^+		Na^+	Na^+
			R^-	
R^-	Cl^-		Cl^-	Cl^-
[1]	[2]		[1]	[2]

Now from thermodynamic considerations it can be calculated, and by actual determination it can be shown, that at equilibrium the product of the concentrations of a pair of diffusible ions (in this case Na^+ and Cl^-) on one side of the membrane will be the same as the product of the concentrations of the same ions on the other side of the membrane. That is,[2]

$$[Na^+]_1[Cl^-]_1 = [Na^+]_2[Cl^-]_2$$

The concentration of the cation Na^+ in (1) must be equal to the sum of anions present $[R^- + Cl^-]$ in order to maintain electrical neutrality, while on the other side the concentration of Na^+ is the same as that of the anion Cl^-. It follows that, in order for the products just mentioned to be equal, the concentration of sodium ions $[Na^+]$ on the left must be greater than on the right and the reverse must be true for the chlorine ions. That is,

$$[Na^+]_1 > [Na^+]_2 \qquad \text{and} \qquad [Cl^-]_1 < [Cl^-]_2$$

Thus it is possible to account for the existence of different concentrations of diffusible ions on the two sides of a semipermeable membrane, the essential condition being the existence of a nondiffusible ion on one side of a membrane which is permeable to the other ions.

This difference in concentration of diffusible ions leads to a difference of potential (electrical pressure, measured in volts) on the two sides of the membrane, and may account for certain electrical phenomena of living matter. The Donnan equilibrium has also been associated with the explanation for a variety of physiological phenomena, such as the formation of gastric juice and other secretions, certain aspects of absorption and elimination, and the differences in concentration of diffusible ions between the blood plasma and cerebrospinal fluid and between the red cells and plasma. In this connection it is well to remember that quantitatively the Donnan equilibrium is based upon ion activity rather than concentration, which was used in the above illustration for purposes of simplicity, and it is not always possible to define precisely the relation between these two properties, particularly in physiological fluids.

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[2] This may also be expressed by the equation for the Donnan equilibrium $x^2 = y(y + z)$ where x is the concentration of Na and Cl ions in the outer solution (2), y the concentration of Na and Cl ions of the free NaCl in the colloidal solution (1), and z the concentration of sodium ions in combination with the nondiffusible anion R. x and y may be determined and z then calculated.

EXPERIMENTS ON DONNAN EQUILIBRIUM

1. *Demonstration of Donnan Equilibrium Using a Membrane.* Introduce 50 ml. of a 2 per cent gelatin solution into one 100-ml. beaker and 50 ml. of distilled water into another. Add 1 ml. of a 0.04 per cent thymol blue solution to each. To the water add 0.1 N hydrochloric acid solution cautiously, drop by drop, until the indicator has a color intermediate between yellow and pink (pH about 2). Add the acid to the gelatin solution until a similar shade of color is obtained (several ml. may be required). The two solutions now have the same hydrogen-ion concentration. Transfer the gelatin solution to a dialyzing bag and suspend it in the beaker containing the water solution. Let it stand. The gelatin solution will gradually turn yellow (decreased acidity) and the outside solution will turn pink (increased acidity), indicating a diffusion of hydrogen ions through the membrane. Do any other ions diffuse? What is the sign of charge on the nondiffusible gelatin ion in this experiment? Explain the results in terms of the Donnan equilibrium.

2. *Demonstration of Donnan Equilibrium without Use of a Membrane.* Prepare 100 ml. of a 5 per cent solution of gelatin. Add 1 ml. of a 0.5 per cent solution of brom thymol blue. Add 0.5 per cent NaOH drop by drop until a color intermediate between yellow and blue (pH about 7) is obtained. Pour it into a 250-ml. beaker and allow it to solidify. Over the gel pour 100 ml. of distilled water containing the same amount of indicator and brought to the same pH. Let it stand, and note the changes in the color of both gel and solution. Explain. Is the presence of an extraneous membrane essential for the development of the Donnan equilibrium?

CHROMATOGRAPHY

Chromatography is the name originally applied by the Polish botanist Tswett in 1906 to a procedure for separating a mixture of different-colored pigments (chlorophylls and xanthophylls) from each other. Tswett found that if the mixture of pigments in petroleum ether as solvent was poured onto the top of a column of calcium carbonate firmly packed in a narrow glass tube, and allowed to drain down the column, the pigments were separated and appeared as colored zones along the column. By the addition of fresh solvent the zones could be further separated and the separate pigments identified under suitable conditions. Tswett called such a column a *chromatogram* and the process the *chromatographic method*.

As has happened with many other fundamental discoveries, the usefulness of Tswett's simple procedure was not generally appreciated until years later. At the present time chromatographic procedures of one kind or another are probably more widely used in biochemical research than any other single type of procedure. Tswett ascribed the differences in the behavior of pigments placed on his columns to differences in adsorbability, the more strongly adsorbed chlorophylls, for example, displacing the weakly adsorbed xanthophylls and thus forcing the xanthophylls further along the column under the influence of the flowing solvent. There are however instances which are more readily explicable on some other basis. For example, a mixture of amino acids placed on a column of starch or powdered cellulose may be chromatographed and separated by the use of certain solvents, but the order of separation of the various compounds present parallels their distribution coefficients (see p. 24) between the solvent and water. Since starch or cellulose will take up appreciable amounts of water[3] it is reasonable to assume that the amino acids dissolve in the water of the column and are extracted from it by the flowing solvent in proportion to their relative solubilities in the two phases (*partition chromatography*). In columns containing ion-exchange resins (see below), it is believed that the functional groups of the resin react directly with the various substances placed on the column, but to a different degree. Thus a resin containing acidic functional groups may be expected to react with a mixture of bases placed on the column in the same way that an acid in solution distributes itself between various bases present. In such a column the bases will be distributed along the column, the most reactive base at the top, and upon elution they will emerge from the column at different rates for similar reasons. There are therefore various explanations for the behavior of substances undergoing chromatographic separation, and the science is still far behind the art in this field. Columns satisfactory for particular purposes are ordinarily found by trial; the explanation usually comes later.

Suitable columns have been made from the various insoluble alkaline-earth phosphates and oxides, starch and cellulose, and ion-exchange resins. The dimensions of the column may vary, but in general a column resembles the common volumetric buret in ratio of length to width. Every column has an inherent capacity for the particular substances being fractionated on it; if this capacity is exceeded, separation will be unsatisfactory. If the various substances on the column can be released by solvent change and thorough washing, a used column can be regenerated and used again. The solvent may flow down the column by gravity or more commonly under a controlled pressure head. The rate of flow of solvent may vary; in general, the more slowly the solvent flows, the more thoroughly equilibrium is reached and the potentialities of the column are realized. The solvent may be aqueous or nonaqueous as required, and may be changed during a separation to modify distribution along the column. The wide variety of solvents available is one of the outstanding advantages of chromatography.

The applicability of chromatography is not limited to colored compounds; thus the name is somewhat misleading. Any procedure which identifies a substance may be used to locate it in the column. Fluorescence under ultraviolet light has been used, as has radioactivity, in cases in which the substance under study is labeled with a radioactive isotope. The column may also be extruded from the tube and cut into sections, and each section may be extracted and analyzed for the substance sought. An improved isolation procedure now widely used is based upon the continuous passage of the pure solvent down the column, the effluent solvent being collected in separate fractions and the fractions analyzed for the material under study. The solvent may be varied during this process, to promote the release of particular compounds from the column. Under carefully controlled conditions it is found that a particular compound may show sufficient reproducibility with regard to the fraction of the effluent in which it is found to afford a tentative basis for identification; this procedure has been used by Moore and Stein in their analysis of the amino acids present in protein hydrolyzates.

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[3] According to the International Critical Tables, the water content of saturated cellulose is 22 per cent on a dry weight basis.

Ion-exchange Resins. The use of ion-exchange resins in chromatography and for other purposes in biochemistry and medicine is of sufficient importance to justify brief consideration at this point. *Ion-exchange resins* are highly insoluble synthetic polymers containing accessible (i.e., titratable) functional groups which are either acidic ($-COOH$, $-SO_3H$) or basic ($-NH_2$, $-\overset{+}{N}(CH_3)_3$, etc.). Resins with acidic functional groups, such as Dowex 50, Amberlite IR 100, and Permutit H, are known as *cation-exchange resins;* those with basic functional groups, such as Dowex 1, Amberlite IR 4, and Permutit S, are the *anion-exchange resins.*

The action of resins is best understood by considering them to be polyfunctional acids or bases which happen to be insoluble. Thus if a solution containing an amine salt is placed on a column packed with a cation-exchange resin containing replaceable hydrogen, the resin will take up the amine cation from the solution and replace it in solution by the hydrogen ion (*hydrogen cycle*). The amine may be subsequently displaced from the resin and washed out of the column by the use of a more acid solvent. A mixture of bases on such a column may be released individually by the use of solvents of varying acidity and electrolyte content. In a similar manner, a cation-exchange resin whose hydrogen has been replaced by sodium will function in a sodium cycle toward other cations such as calcium and magnesium. A solution containing a calcium salt will emerge from such a column as a solution of the sodium salt, the calcium remaining attached to the resin. The resin may be returned to its original state by treatment with a large excess of sodium ions. This is a principle used in the softening of hard water. An anion-exchange resin which has been saturated with chloride ion will function in a chloride cycle towards other anions; a column containing such a resin will remove weak organic acids from solution as anions and replace them with chloride.

The use of ion-exchange resins is not confined to chromatography, but is acquiring increasing importance in other aspects of biochemistry and medicine. Examples of such uses include the use of resins to decalcify blood and thus prevent blood clotting; clinical use to control gastric acidity and the dietary absorption of electrolytes from the intestinal tract by the oral administration of suitable resins; and the demineralization of milk for special dietary purposes. Since it is now known that resins can be "tailor made" to fit almost any desired purpose, such clinical use of resins would appear to be on the increase.

Paper Chromatography. A remarkable development in the field of chromatography which is perhaps unequalled in the field of biochemical procedures for its simplicity, versatility, and widespread application is the use of filter paper for chromatographic purposes by a procedure first described by Consden, Gordon, and Martin.[4] The basic principle is very simple. A small drop of solution containing the mixture of compounds it is desired to separate is evaporated to dryness on a piece of filter paper, and a suitable solvent is allowed to flow slowly along the filter paper over this spot, either by gravity (descending) or capillarity (ascending). The substances in the initial spot are extracted by the flowing solvent and carried along the filter paper to an extent which is related to their distribution between the solvent and the water phase of the filter paper, as discussed previously. After the solvent has flowed for a suitable distance along the paper, the paper is removed and freed of excess solvent by quickly drying, and suitable tests are applied to the filter paper to locate the various compounds under study. Under the proper conditions it is found that each substance present has been carried away from the initial spot to a characteristic extent and is localized in a relatively small area on the filter paper.

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[4] Consden, Gordon, and Martin: *Biochem. J.*, **38**, 224 (1944).

The simplest expression of the response of a particular compound to this procedure is the so-called R_F value, which is the ratio of the distance the compound moves along the paper to the distance covered by the solvent. Thus if the solvent front is 12.0 cm. from the spot of application of the compound, and the compound is located 9.0 cm. from this spot, the R_F value is 9.0/12.0, or 0.75. The absolute value of R_F for a particular compound is of course dependent upon such factors as the nature of the solvent, the temperature, and the presence of other substances which influence the distribution of compound between the solvent and water. With suitable attention to these details, however, the R_F value for a pure substance is found to be sufficiently reproducible to serve as an approximate basis for identification.

An improved procedure is the so-called two-dimensional chromatography. Here the spot containing the substances under study is dried in one corner of a square of filter paper. The solvent, running as before, carries the substances along one edge of the paper in proportion to their R_F values for that solvent. After the excess solvent is removed by drying, the paper is turned 90° and a second solvent is allowed to flow across the paper at right angles to the direction of flow of the first solvent and in such a way as to carry the various substances present from the edge of the paper to the middle regions. After the second solvent is removed by drying, the substances are located by suitable means. By the selection of suitable solvents it is possible using this procedure to separate and identify the components of even such complex mixtures as the various amino acids present in a protein hydrolyzate (see Frontispiece), using only small amounts (a few milligrams) of material and ordinary laboratory equipment.

There are of course many refinements to the procedures described here. By the careful use of the technique, the compounds separated are usually localized in a small and reproducible area of the filter paper. If the compound is detected at this spot by a color reaction, measurement of the color intensity may be used to determine the amount of material present. The area may also be cut out and the compound extracted from the paper, thus essentially isolating it in pure solution, for confirmation of its chemical nature by other tests. The student will find many applications of paper chromatography to biochemical problems in this book and in the biochemical literature.

EXPERIMENTS ON CHROMATOGRAPHY

1. *One-dimensional Paper Chromatography.*[5] For demonstration purposes solutions of amino acids will be chromatographed in this experiment. The amino acids will be located on the filter paper by reaction with ninhydrin to give a purple color. Solutions of glycine, aspartic acid, and a mixture of glycine and aspartic acid, all 0.03 M, will be used. If desired, a fourth solution containing either or both amino acids can be used as an unknown.

Cut strips of Whatman No. 1 filter paper to the dimensions 13.5 × 1.8 × 1.0 cm. Pierce a small hole in each strip, in the center and about 4 mm. from the broad end. This will be used for hanging the strips during the drying process. To minimize contamination, avoid touching the strips with the fingers.

For each solution under examination, draw a portion into a capillary pipet having a very fine tip,[6] and apply the tip of the pipet quickly and lightly to a filter-paper strip at the center and about 6 mm. from the narrow end. By this procedure a volume of approximately 0.2 μl. of solution is deposited on the paper to form an area which should not be in excess of 1.5 mm. in diameter. Circle the wet area with a light pencil line and allow to dry. Prepare a separate strip for each solution under examination.

Place approximately 0.5 ml. of water-saturated phenol[7] in the bottom of a 6-inch test tube. Using one tube for each strip, insert the narrow end of the treated paper strip into the phenol solution in such a way that the strip does not touch the walls of the tube except at the top. Stopper the tube with a soft cork. Allow to stand undisturbed at room temperature for 2 to 3 hours or until the solvent has ascended by capillarity to within about 5 mm. of the hole at the top of the strip. Remove the strip, place a bent wire or paper clip in the hole, and suspend in a drying oven at 110° for about 3 minutes. Remove, and spray the entire strip lightly with a solution of ninhydrin contained in an atomizer.[8] Replace in the oven and dry for about 4 minutes.

With a pencil, mark the edge of the solvent front on the strip, and encircle any colored spots present. Mark the approximate center of each spot. Measure the distance from the original spot where the solution was applied to the edge of the solvent front and to the center of each colored area. Calculate the R_F value for each colored spot, where R_F is the ratio of the distance traveled by the material in the spot to the distance traveled by the solvent. How do your values compare with those given by Rockland and Dunn (glycine, 0.49; aspartic acid, 0.25)? From the volume of solution used, and the molar concentration of amino acid, calculate the actual amount of amino acid separated and identified by this procedure.

2. *Two-dimensional Paper Chromatography.* Obtain a sheet of filter paper about 17 cm. square (Whatman No. 1 or Schleicher and Schull No. 597 are satisfactory). Using a capillary micropipet place a small drop of amino acid solution (1–2 μl., containing 10–20 μg. of amino acid) in one corner of the paper, about 2 cm. from each edge. Circle the wet area with a light pencil line and allow to dry. Roll the paper into a cylinder and staple the edges together.

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[5] Procedure of Rockland and Dunn: *Science*, **109**, 539 (1949).

[6] Draw 5-mm. pyrex capillary tubing to about 2 mm., cut into suitable sizes, and grind the tip to about 0.5 mm. with 400–600 mesh carborundum. These pipets deliver about 0.2 μl. when used as described. Micropipets may be obtained from Microchemical Specialties Co., 1934 University Ave., Berkeley, California.

[7] Add 25 ml. of distilled water to 75 g. of pure phenol. Warm to bring about solution. A pink color is not objectionable.

[8] Dissolve 0.25 g. of ninhydrin in 100 ml. of water-saturated butanol.

Do not overlap the edges, but allow the staples to serve as links. Stand the cylinder upright, with the applied spot at the bottom, in a 10-cm. petri dish filled to a depth of about 0.5 cm. with a suitable solvent (see below). Cover the assembly with a bell jar or battery jar, preferably with a tightly fitting glass plate as a base. Allow to stand until the solvent has risen to within a few centimeters of the top of the cylinder. Remove from the petri dish, dry quickly in an oven, and unroll. Remove the staples, turn the paper so that the circled spot is in the lower right corner, and make a new cylinder as before. Place upright in a petri dish containing a second solvent, cover with a bell jar, and let stand as before. When the solvent has risen to within a few centimeters of the top of the paper, remove from the petri dish and dry quickly in an oven. Unroll, spray the dried paper uniformly with ninhydrin solution, and dry as described under Exp. 1. Circle the colored areas with a pencil line to mark the spots, since the colors will fade in a few days.

Try this experiment with solutions of various amino acids and with various solvents. Suitable solvents[9] include (1) water-saturated phenol (Exp. 1); (2) a mixture of 70 parts *n*-propanol and 30 parts water; (3) a mixture of equal parts of 2,4,6-collidine, and 2,4-lutidine to which one-third volume of water is added; (4) a mixture of *tert.*-butanol, water and 85% formic acid in the proportions of 69.5:29.5:1.0; (5) water-saturated phenol made alkaline with 1% concentrated NH_4OH. How do your results compare with those shown in the frontispiece?

***3. Detection of Glutamate in Canned Foods.* An interesting use of the procedure described in Exp. 1 has been described by Patton and Foreman[10] for the detection of added sodium glutamate in processed foods. Sodium glutamate is an approved flavoring agent which may or may not be added to canned foods such as chicken soup. Try the procedure of Exp. 1, using fluid from commercial canned chicken soup or meat sauce. As a control, run an 0.03 M solution of sodium glutamate. As a second control, glutamate may be added directly to the solution under test. Do your results indicate the presence of glutamate in the food tested?**

***4. Column Chromatography.* Experiments illustrating the use of column chromatography will be found in Chapters 7 and 10.**

OSMOTIC PRESSURE

Membranes which permit the passage of molecules of the solvent but not of the molecules or ions of a substance in true solution are known as *semipermeable membranes.* One type of semipermeable membrane is made by depositing copper ferrocyanide in the walls of a porous porcelain cup. If such a cup is filled with a solution of sugar in water and is then immersed in water, the sugar molecules cannot pass through the membrane. The water molecules, however, permeate readily and the level of the solution in the cup will rise. The pressure that must be exerted upon the solution within the cup to prevent any increase in volume is a measure of the osmotic pressure of the solution. The magnitude of osmotic pressure is indicated by the fact that a 10 per cent solution of cane sugar at 25° C. has an osmotic pressure of 7.6 atmospheres.

Whatever may be the fundamental cause of osmotic pressure there is no question but that it appears to be, for substances in solution, analogous to the pressure of gaseous molecules. Thus 1 gram-molecular weight of an ideal gas at 0° C. and in a volume of 1 liter exerts a pressure of 22.4 atmospheres; 1 gram-molecular weight of an ideal nonelectrolyte in solution under the same conditions has an osmotic pressure of 22.4 atmospheres. Furthermore under ideal conditions osmotic pressure is, like gas pressure, directly proportional to the absolute temperature and to concentration, and is independent of the chemical nature of the dissolved material. Thus equimolecular concentrations of all nonelectrolytes have the same osmotic pressure, or are isosmotic. It is therefore possible to determine molecular weight by the measurement of osmotic pressure, and this procedure has found considerable application in biology.

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[9] Underwood and Rockland: *Food Res.*, 18, 17 (1953).

[10] Patton and Foreman: *Food Tech.*, 4, 83 (1950).

The osmotic pressure of electrolytes is considerably higher than that of equimolar solutions of nonelectrolytes, since an ion is theoretically as effective osmotically as a molecule. For example, a solution of NaCl containing Na^+ and Cl^- ions should have twice the osmotic pressure of an equimolar solution of nonelectrolytes such as glucose or urea. By actual measurement it is usually found that the osmotic pressure of electrolytes is somewhat less than the expected value, presumably because of differences between the osmotic effectiveness (*activity*) of the ions and their concentration. The relation between ion concentration and activity must be considered in any calculation of osmotic pressure for solutions containing electrolytes.

A colloidal particle is as effective osmotically as a molecule or ion. Colloidal solutions therefore have very low osmotic pressures compared to equal weights of substances in true solution because of the large size and relatively small number of particles present. Colloidal osmotic pressures, although small, are nevertheless very important in biology, since colloids do not ordinarily diffuse through membranes and only substances which are impermeable to a membrane ordinarily can influence the osmotic flow of fluid across the membrane. For example, the osmotic pressure of the blood colloids is only about 0.5 of 1 per cent of the total osmotic pressure of blood, yet the blood colloids become the determining factor in the flow of fluid by osmosis across those physiological membranes which are freely permeable to all other dissolved substances present.

The maintenance of the normal water content of plant and animal cells is determined largely by osmotic pressure relationships. Cells placed in contact with a medium which has a lower osmotic pressure than the cell contents will absorb water (swell), the extent of swelling depending upon the osmotic-pressure difference between cell contents and medium. Such a medium is said to be hypotonic to the cell. A hypertonic solution will bring about the reverse process, the cell losing water and diffusible solutes to the medium, and shrinking. If the medium causes no change in cell volume, it is isotonic to the cell. In relating these phenomena to the osmotic pressures of the cell contents and of the surrounding medium, for reasons given above it is essential that permeability of the cell membrane to the solutes present be taken into consideration.

Osmotic pressure may be measured by the use of a semipermeable membrane as described previously and illustrated by the experiments which follow; but for practical reasons associated with the enormous pressures concerned and the difficulty in obtaining a truly semipermeable membrane, other methods are usually employed for all except colloidal solutions. These methods make use of those other physical properties of solutions which, like osmotic pressure, depend upon the number of dissolved particles (e.g., vapor pressure, rise in boiling point, or depression of freezing point of the solvent). An aqueous solution with an osmotic pressure of 22.4 atmospheres, for example, has a freezing point 1.86° C. below that of pure water, with other values in direct proportion. The freezing-point method is by far the most widely used in investigations on osmotic pressure in biological fluids.

EXPERIMENTS ON OSMOTIC PRESSURE

1. *Demonstration of Osmotic Pressure of a True Solution (Cane Sugar)*. Prepare a dialyzing bag similar to that described on p. 10. Tie the mouth of the bag to a one-hole rubber stopper. With a pipet fill the bag with a 10 per cent solution of cane sugar. Insert a glass tube (about 30 cm. long and of 1 to 2 mm. bore) through the hole in the stopper until the end dips below the surface of the fluid in the bag. Blow gently through the tube to create a slight positive pressure in the bag. Support the bag in a large beaker of water with the glass tube in an upright position and the level of fluid in the bag and beaker approximately the same. Observe the rise of fluid in the glass tube. Ultimately the sugar will diffuse through the membrane and the solution level in the tube will fall, but meanwhile the existence of an osmotic pressure will be demonstrated. Copper ferrocyanide membranes deposited in porcelain cups are truly semipermeable, and are necessary for quantitative determination of the osmotic pressure of true solutions by this method.

2. *Demonstration of Osmotic Pressure of Colloidal Solutions*. The bag employed in Exp. 1, or a similar one, may be used and the determination is made in the same way. The highest point of the column is generally reached in five or six hours. Membranes of this type are not permeable to proteins, so a true measure of their osmotic pressure may be obtained. The pressures attained, however, are very much lower. A 1 per cent solution of gelatin may give a rise of 25 to 50 mm.

3. *Osmotic Pressure of Red Blood Cells*. Add a drop or two of blood to 5 ml. of each of the following solutions: 0.3, 0.9, and 5.0 per cent sodium chloride. Examine each suspension under the microscope. Explain the findings. Calculate the molar concentration and approximate osmotic pressure of each of the three solutions. What is the osmotic pressure of the red-cell contents?

Repeat, using the following solutions: 0.16 M sodium chloride, 0.32 M urea, and 0.32 M urea containing 0.16 M sodium chloride. Observe as above. Calculate the osmotic pressure of these solutions. Which are isosmotic with the red cell? Which are isotonic? Explain.

SURFACE TENSION

The molecules in the interior of a liquid are surrounded by other molecules on every side and are thus subject to uniform attractions in every direction. On the other hand the molecules in the surface are attracted inward and by other molecules in the surface, but there is little attraction outward because there are so few molecules outside. The result of this inward attraction is that the number of molecules in the surface tends to be reduced to a minimum and the surface contracts until its area is the smallest possible for a given volume of liquid. This explains the tendency of small drops of liquid to assume a spherical form. This spontaneous contraction of a liquid surface indicates that there is a certain free energy in the surface, and that in order to extend this surface by bringing molecules into the surface a certain amount of work must be done in overcoming the attraction of the other molecules in the interior. It is customary, in considerations involving surface forces, to substitute for the free surface energy the concept of a surface tension acting in all directions parallel to the surface. Thus, if the surface tension of a liquid is γ dynes per cm., the work done in increasing the surface area by 1 sq. cm. will be γ ergs, and the free surface energy of the surface will be γ ergs per sq. cm.

When two immiscible liquids are in contact with each other, the dividing surface or interface also contracts spontaneously because each type of molecule in the surface is attracted by its own kind of molecule in the interior. Thus the interface also possesses a free surface energy, or a surface tension known as the interfacial tension. When the interfacial tension between two liquids is zero, the two liquids are completely miscible.

An intimate mixture of two immiscible liquids is known as an *emulsion*, one liquid being dispersed in the form of droplets in a continuous or external phase of the other liquid. Emulsification is facilitated by the presence of substances called emulsifying agents, which minimize the tendency of the droplets to coalesce. Among the most effective of such agents are substances which lower surface tension, such as soaps, proteins, bile salts, etc. The action of bile salts in lowering surface tension is an important aid in the emulsification and digestion of fats in the intestinal tract.

Emulsions may be of either the oil-in-water or the water-in-oil type, depending upon which liquid is the continuous phase. Sodium soaps, which are more soluble in water than in oil, promote the formation of oil-in-water emulsions; calcium or magnesium soaps, which are oil-soluble, promote the formation of water-in-oil emulsions. This has been offered as an explanation for certain antagonistic effects of Na^+ and Ca^{++} ions on the physical state of protoplasm.

EXPERIMENTS ON SURFACE TENSION

1. *Demonstration of Surface Tension.* Half fill a large thoroughly cleaned and washed beaker with fresh distilled water. Sprinkle some small particles of camphor over the surface. The particles will move about rapidly on the surface. (Camphor lowers the surface tension of water.) The tension of the surface film will be lowered more rapidly on one side of the particle than on another because of more rapid solution of the camphor at some points, and the particle will be pulled in the direction of greater tension. Touch a glass rod to a cake of soap and then to the surface of the water. The surface tension is greatly reduced and the particles instantly stop moving. Rinse the beaker thoroughly and repeat, but touch the glass rod to the oily skin of the face and then to the water surface. (Oil also reduces surface tension.) The particles will move away from the rod (point of low tension) and will finally stop moving. It has been calculated that a layer of oil one molecule deep is sufficient to produce this effect.

2. Determination of Relative Surface Tension by Drop Method. The surface of a liquid tends to contract so as to produce a minimum area. When flowing through a small opening it therefore tends to form drops which fall away when gravity overcomes the surface tension. The number of drops formed from a given weight or volume of fluid varies, therefore, with the surface tension.

Procedure. Set up a Traube stalagmometer as in Fig. 3 so that the whole may be immersed in a constant-temperature bath. For approximate comparative results an ordinary 1- or 2-ml. pipet clamped in a vertical position may be used, regulating the outflow by the use of the finger or a piece of rubber tubing with attached screw clamp so that discrete drops are formed at a uniform rate. Fill it with water above mark *A*. When the level of the water falls to this mark, start counting the number of drops that fall until mark *B* is reached. By means of the scale divisions above and below *A* and *B* (knowing how many divisions correspond to a drop), fractions of a drop may be estimated. If γ represents the relative surface tension of the liquid tested, N_W the number of drops of water, N the number of drops of the unknown, and S its specific gravity, $\gamma = \frac{N_W \cdot S}{N}$. Determine the surface tensions of water, of a very dilute soap solution, and of olive oil, cleaning the instrument thoroughly between tests, and rinsing several times with the solution tested before taking readings. Olive oil has a lower surface tension than water. Soaps markedly lower the surface tension of aqueous solutions.

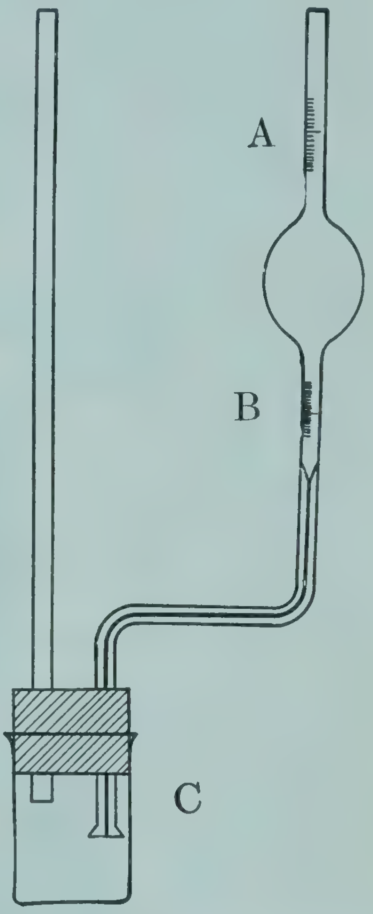

FIG. 3. STALAGMOMETER.

3. Determination of Surface Tension by Ring Method of Du Noüy.[11] The drop method for determining surface tension described in Exp. 2 is a static method. So also is the measurement of the rise of fluid in a capillary tube. The oscillating-jet method is a dynamic method in that new surfaces are constantly being formed. All methods give practically concordant results in the case of pure liquids, but not in the case of solutions.[12] Du Noüy has suggested a static method based on the measurement of the force required to overcome the adherence of a platinum ring to the surface of the liquid in question. The apparatus consists of a platinum ring hanging from a lever arm which can be raised by applying torsion to the wire to which it is fixed. The torsion is measured on a scale which can be calibrated directly in dynes per cm., the absolute unit of surface tension. Determinations of surface tension by this method may be accurately made on 1-ml. portions of fluid in 20 to 30 seconds. The method is particularly useful in physiological studies.[13] The surface ten-

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[11] Du Noüy: *J. Gen. Physiol.*, **1**, 521 (1919). This method has been criticized on the ground of inaccurate application of the theory, and an improved technique suggested. Harkins and Jordan: *Science*, **72**, 73 (1930).

[12] For a comparative study of different methods, see Ferguson: *Trans. Faraday Soc.*, **17**, 370 (1922).

[13] The Du Noüy surface-tension apparatus, with detailed directions for operation, may be obtained from houses supplying chemical and physical apparatus. A tensiometer of the ring type designed so that a measurable downward force as well as an upward force can be exerted, and which is therefore more generally useful, may be obtained from the Central Scientific Company, Chicago.

sion of a resting surface of serum diminishes on standing (*time drop*) due to the gradual accumulation of surface active substances (see p. 22). This phenomenon has been investigated by Du Noüy.[14] Using this apparatus, determine the surface tension of the liquids tested in Exp. 2 and compare the results obtained by the two methods (see Fig. 4). This apparatus has been modified to permit the measurement of interfacial tension between two liquids.[15]

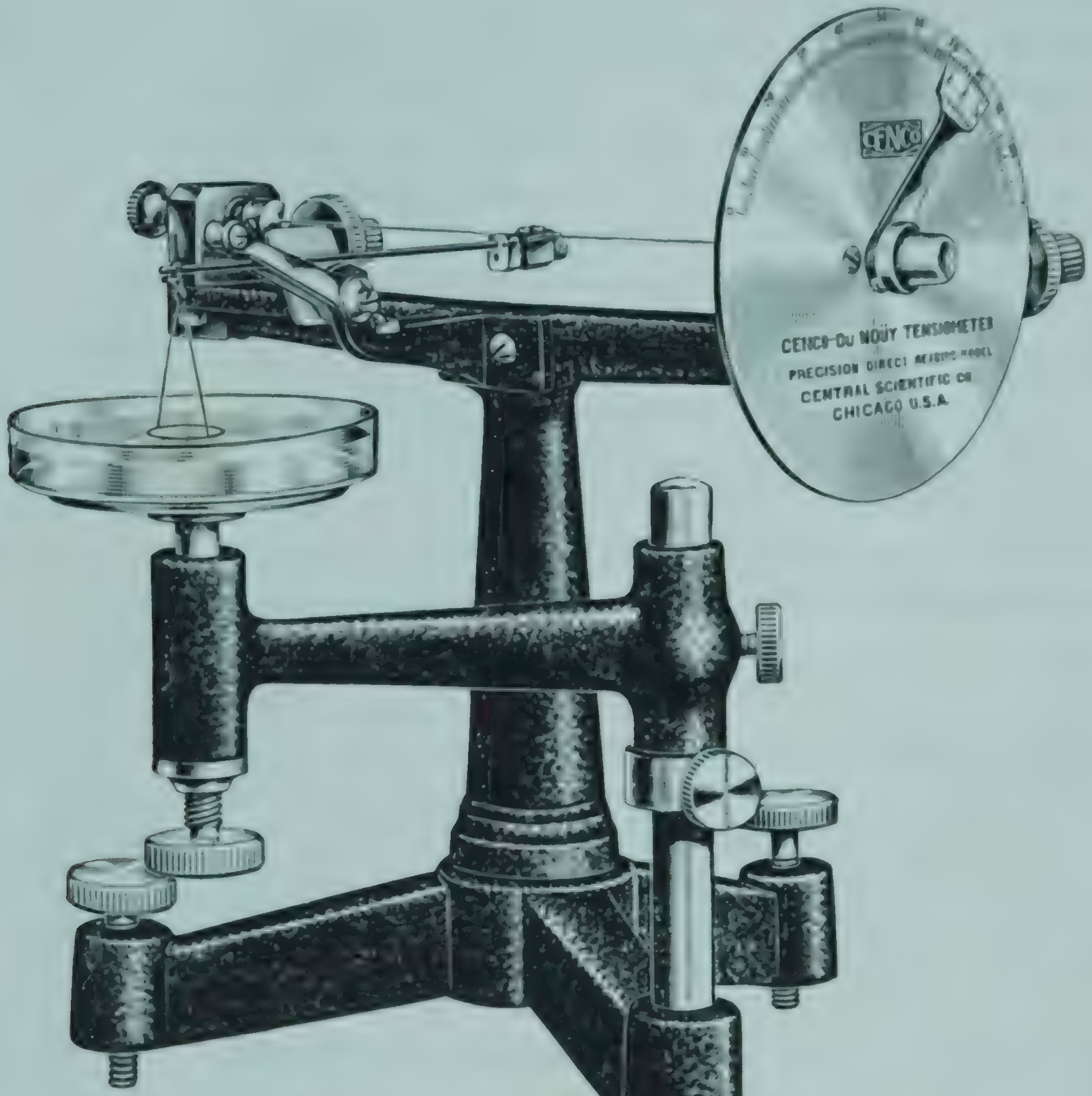

FIG. 4. DU NOÜY TENSIOMETER.

DISTRIBUTION OF DISSOLVED SUBSTANCES BETWEEN LIQUID PHASES

If a substance is soluble in each of two liquids which are immiscible and in contact with each other, it is found that at equilibrium the substance is distributed between the two liquid phases in a characteristic manner. The numerical expression of this property is the *distribution coefficient* (sometimes called *partition coefficient*) K, which is defined as the ratio at equilibrium of the concentration C_A of substance in phase A to its concentration C_B in phase B, i.e. $K = C_A/C_B$. It is clear that K is characteristic for a particular set of conditions only, since its value will be influenced by any factor affecting solubility in either phase, such as temperature, pressure, the presence of other solutes, the effect of various solvents on association or dissociation of solute, etc.

The distribution of biologically important substances between "water" and "oil" phases of protoplasm has attracted considerable attention. It is not surprising to find that substances which are predominantly lipide-soluble are concentrated in the various fats and oils of living tissues. The presence of fat-soluble vitamins in various fish-liver oils is an example, as is also the reported concentration of the insecticide DDT in the fat of milk obtained from cows exposed to DDT-contaminated areas. The action of drugs has been studied in relation to water-lipide distribution. According to one theory, the relative lipide-solubility of various drugs is of importance in determining penetrability of such drugs into, and action upon, such tissues as the brain and nerves, which are relatively rich in lipide material. In connection with such theories however it must be remembered that the physical nature of the so-called *oil phase* of protoplasm has not been too clearly defined. There are relatively few tissues other than adipose and similar tissue where discrete fat droplets are found within the cell. The lipide material of brain and nerve tissue is not fat or oil but consists of phospholipides and similar compounds (see p. 290) which are dispersed within the primarily aqueous phase of the cell in a way which is not entirely clear, but is known to involve intimate association with the proteins and other water-soluble constituents of the cell. The extent to which the distribution of a drug or other substance between water and peanut or olive oil, as determined in the laboratory, is applicable to the complex environment of the living cell remains to be more clearly defined.

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[14] Du Noüy: *J. Exptl. Med.*, **41**, 779 (1925).

[15] Du Noüy: *J. Gen. Physiol.*, **7**, 625 (1925).

The distribution of dissolved material between immiscible solvents has many practical applications. Widespread use is made of such nonaqueous solvents as ether, chloroform, and benzene for the extraction from biological material of lipides, fat-soluble vitamins, various organic acids, and other similar substances. An important advance in the use of solvent distribution for the isolation and characterization of substances of biological importance has been made by Craig[16] in devising his procedure for *countercurrent distribution.* In this procedure the material under examination is subjected to a unique and systematic series of extractions by an immiscible solvent in a sequence of extraction vessels or tubes in such a way that various substances become separated and distributed among the tubes in a characteristic manner. The region of concentration of a particular substance among the extraction tubes (the *zone* or *band*) is determined largely by the distribution coefficient of the substance between the two phases used, and the number of extractions applied. In the theoretical treatment of countercurrent distribution, the localization of material has been compared to the effect of the various plates in a fractional distillation column. As in such a column, even closely related compounds may be separated from one another by countercurrent distribution. The curve relating the amount of material present to the number of extractions has a characteristic location for a particular substance and may be used for identification purposes. Furthermore, the shape of the curve is predictable on a theoretical basis for a homogeneous substance; any deviation from such a theoretical curve may be construed as indicating the presence of impurities. Countercurrent distribution has found wide application in the isolation and characterization of biologically active substances, comparison of natural and synthetic compounds, and establishment of the purity of drugs and similar substances. An experiment illustrating the principles involved is described below.

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[16] Craig: *J. Biol. Chem.*, **155**, 519 (1944); *Anal. Chem.*, **22**, 1346 (1950).

EXPERIMENTS ON DISTRIBUTION OF DISSOLVED SUBSTANCES BETWEEN LIQUID PHASES

Countercurrent Distribution. 1. This experiment is designed to illustrate the principles of Craig's countercurrent-distribution procedure for the isolation and identification of substances in solution. A study will be made of the distribution of propionic acid between 2.2 M phosphate buffer at pH 5.2 (aqueous phase) and isopropyl ether.[17]

Set up nine 25-ml. graduated cylinders with tightly fitting glass stoppers, and number them from 0 to 8. Place 15 ml. of water-saturated isopropyl ether[18] into each tube. Into Tube 0 place 0.2 ml. of propionic acid. Now add 7.5 ml. of phosphate buffer to Tube 0, stopper, and invert 50 times. Allow the layers to separate. Remove the lower layer as completely as possible[19] and transfer it to Tube 1. Add 7.5 ml. of fresh lower phase to Tube 0. Stopper, equilibrate by inverting both tubes 50 times as before, and allow the phases to separate. Transfer the lower phase of Tube 1 to Tube 2, the lower phase of Tube 0 to Tube 1, and add 7.5 ml. of fresh lower phase to Tube 0. Again invert all tubes 50 times. Continue the transfer of lower phase from each tube to the succeeding tube, the addition of fresh lower phase to Tube 0, and the equilibration, until all nine tubes have equal volumes of both phases. The distribution is now completed within the limits of the number of tubes used.

During this experiment, the volumes of the two phases should be noted occasionally, particularly that of the leading lower phase, and any deficit made up by the addition of fresh lower or upper phase as required, so that the ratio of phases is maintained at 2:1 in each tube. To facilitate equilibration between transfers, it is convenient to clamp the cylinders or tubes to a horizontal rod which can be turned with a crank. Such a device is illustrated in Fig. 5. In place of the glass cylinders, test tubes or separatory funnels may be used.

When the distribution is completed, transfer a 2-ml. portion of each upper phase to separate Erlenmeyer flasks, add 10 ml. of 95 per cent alcohol and 5

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[17] For the use of this system in the separation of various lower fatty acids, see Sato, Barry and Craig: *J. Biol. Chem.*, **170**, 501 (1947).

[18] Solutions required.

Isopropyl ether, c.p.

Phosphate buffer, 2.2 M, pH 5.2. Dissolve 132 g. of KH_2PO_4 and 46 g. of $Na_2HPO_4 \cdot 12H_2O$ in about 400 ml. of warm distilled water, cool, dilute to 500 ml. in a volumetric flask, and mix.

Before use, both the isopropyl ether and the phosphate buffer must be saturated with each other. It is convenient to place the 500 ml. of phosphate buffer into a 1-liter glass-stoppered bottle and then fill the bottle with isopropyl ether. Shake well and allow the phases to separate. As needed, the isopropyl ether may be drawn from the upper phase and the phosphate buffer from the lower phase. Occasional shaking will keep the two phases saturated at room temperature.

Phenolphthalein solution. See Appendix.

Sodium hydroxide, 0.01 N. Dilute 10 ml. of 0.1 N sodium hydroxide solution to 100 ml. with water in a volumetric flask, and mix.

[19] A hypodermic syringe with a long needle or a pipet with a fine tip, attached to a hypodermic syringe by rubber tubing, are satisfactory. The same syringe or pipet may be used for all transfers, and it is not necessary to rinse between transfers.

drops of 1 per cent alcoholic phenolphthalein solution to each flask, and titrate with 0.01 N NaOH solution to the first permanent pink color. As a control, titrate 2 ml. of fresh upper phase in the same way. Subtract the buret reading for the control from that for each tube. Plot milliliters of alkali required for each tube against tube number, on cross-section paper, and draw a smooth curve to include all the points. This is the distribution curve for the particular solute and solvent pair used.

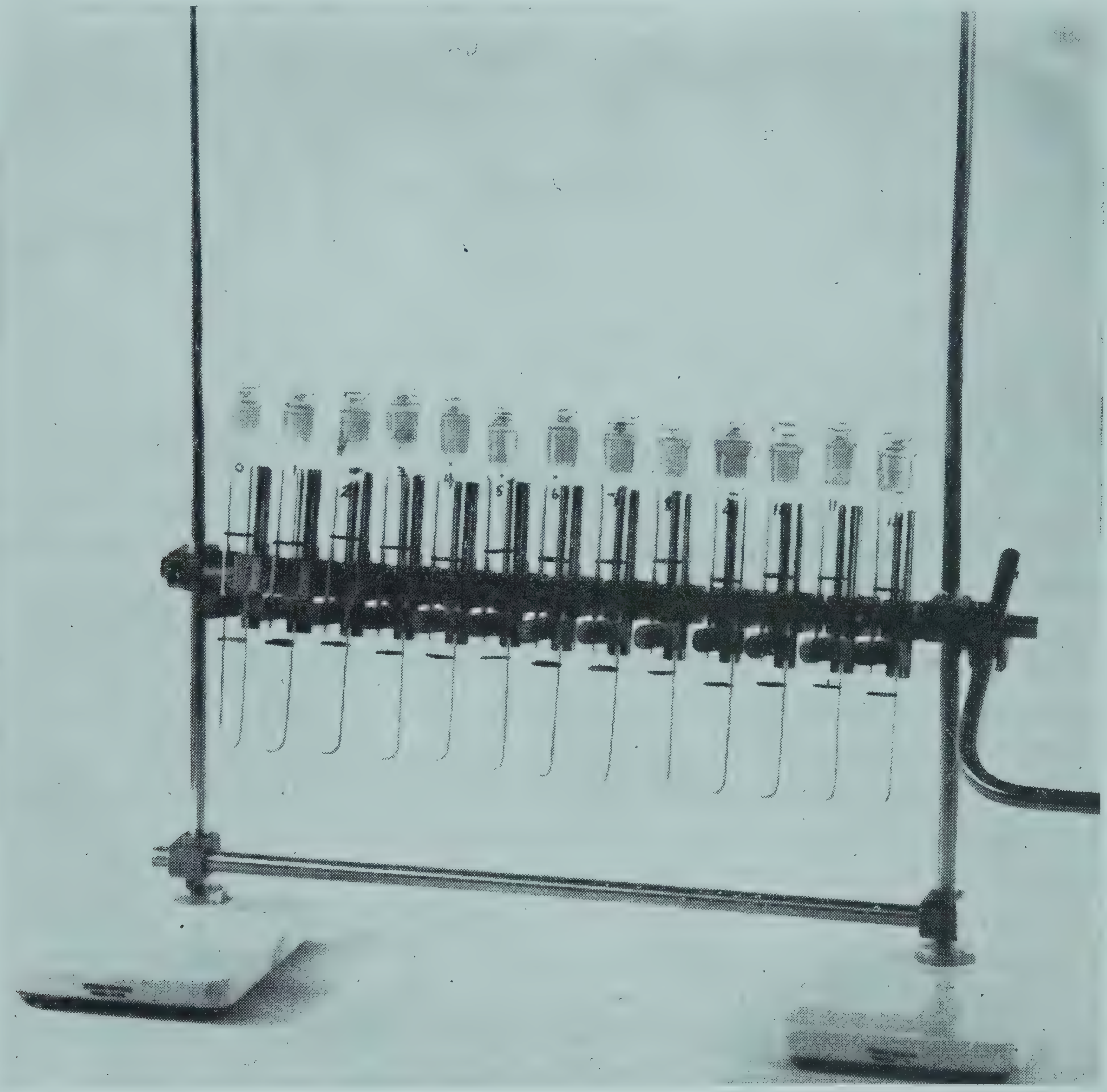

FIG. 5. COUNTERCURRENT DISTRIBUTION INVOLVING 12 TRANSFERS.

Courtesy, L. C. Craig and D. Craig: "Extraction and Distribution," in *Technique of Organic Chemistry*, Vol. III, A. Weissberger, editor. Copyright 1950, Interscience Publishers, New York-London.

CALCULATION. The approximate value of the distribution coefficient K may be calculated from the formula:[20]

$$N = n \times \frac{1}{1 + rK}$$

where N is the tube number at the peak of the curve, n is the number of transfers, i.e. the number of tubes minus 1 (in this experiment equal to 8) and r is the ratio of upper-phase volume to lower-phase volume, i.e., $r = 2.0$. How does your value compare with the value of 0.50 estimated by Sato *et al.*?

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[20] This equation is derived from the original equation $N = n \times \frac{K}{K+1}$ which was developed for movement of the upper phase, with equal volumes of the two phases present. When the lower phase moves, and the ratio r of upper-phase volume to lower-phase volume is other than 1.0, K in the original equation is replaced by $1/Kr$, and the equation in the text is obtained. For a discussion of the limitations of these equations, see Williamson and Craig: *J. Biol. Chem.*, **168**, 687 (1947).

Another method for calculating the value of K is based on the fact that the ratio of the concentrations of substance analyzed for in any two tubes is related to K as follows:

$$K = \frac{1}{F} \times \frac{T_N}{T_{N-1}}$$

where T_N and T_{N-1} refer to the amounts of material (or aliquots thereof, since ratios are concerned) found in tube N and in the tube immediately preceding it in the countercurrent distribution. F is a factor, differing for each tube,[21] with the following values in this experiment: $N = 2$, $F = {}^7/_2$; $N = 3$, $F = {}^6/_3$; $N = 4$, $F = {}^5/_4$; $N = 5$, $F = {}^4/_5$; $N = 6$, $F = {}^3/_6$. Again replacing K by $1/Kr$ for reasons already given,[20] the equation as applied here is:

$$K = \frac{1}{r} \times F \times \frac{T_{N-1}}{T_N}$$

Using this equation, calculate K when N equals 2, 3, 4, 5, and 6. Average your results. How do they compare with the value obtained above?

2. Repeat this experiment using (a) acetic acid ($K = 0.09$) and (b) butyric acid ($K = 2.24$). How do the curves and calculated results compare with those for propionic acid?

HYDROGEN-ION CONCENTRATION

Acids and Bases: Hydrogen-ion Concentration and Titratable Acidity. Acids may be defined as compounds which yield positively charged hydrogen ions[22] in solution; bases, as compounds which yield negatively charged hydroxyl ions in solution. Strong acids, such as HCl, are completely ionized at all concentrations; they therefore have a hydrogen-ion concentration which is equal to the concentration of acid present. Weak acids, such as acetic acid, exist in solution largely in the molecular or undissociated form, and have a hydrogen-ion concentration under ordinary conditions which is small relative to the total acid concentration. A similar distinction is made between strong bases and weak bases. Most neutral salts are considered to be completely ionized in solution.

When acids react with bases a double decomposition occurs which results in the formation of a salt and water, as in the following equation.

$$\begin{array}{ccccc}
\text{HA} & \rightleftarrows & \text{H}^+ & + & \text{A}^- \\
\textbf{(Acid)} & & & & \\
 & & & & \\
\text{BOH} & \rightleftarrows & \text{OH}^- & + & \text{B}^+ \\
\textbf{(Base)} & & \downarrow\uparrow & & \uparrow\downarrow \\
 & & \text{H}_2\text{O} & & \text{BA} \\
 & & \textbf{(Water)} & & \textbf{(Salt)}
\end{array}$$

---

[21] For the derivation and significance of this factor, see Williamson and Craig, *loc. cit.*

[22] According to modern concepts of atomic structure the hydrogen ion is a hydrogen atom which has lost an electron, leaving a proton, with the symbol H^+. Until relatively recently this symbol was commonly accepted to signify the *hydrogen ion in aqueous solution.* There is considerable evidence, however, that the proton and the hydrogen ion are not identical, and that the hydrogen ion is a proton which has reacted with water to form an ion such as H_3O^+. For purposes of simplicity the symbol H^+ will continue to be used throughout this book to signify the hydrogen ion, since the theoretical treatment as presented is independent of the symbol used.

Such reactions, known as neutralization reactions, go to completion because the water formed during the reaction is itself so feebly ionized that its formation leads to the removal of practically all of the hydrogen and hydroxyl ions from solution. The amount of base required to neutralize a definite volume of acid (i.e., the titratable acidity) depends entirely upon the concentration of the acid and is independent of its degree of dissociation. This fact becomes evident upon study of the above equation. As the base is added some of the hydrogen ions in the solution combine with the added hydroxyl ions to form water, and are thus removed from solution. This removal of hydrogen ions disturbs the equilibrium between the undissociated molecules of acid and its ions, and more of the acid dissociates in an attempt to restore this equilibrium. If sufficient base is added this process will continue until all of the acid has been dissociated and neutralized. This will be the case irrespective of whether the acid was originally highly ionized or feebly ionized—i.e., whether it was a strong acid or a weak acid. On the other hand, the hydrogen-ion concentration of an acid—i.e., the actual amount of free hydrogen ions present in the solution at a particular time—depends not only upon the concentration of the acid but also, for weak acids, upon the degree of dissociation. Thus the hydrogen-ion concentration of normal hydrochloric acid, which is 100 per cent ionized, is approximately 100 times as great as the hydrogen-ion concentration of normal acetic acid, in which less than 1 per cent of the molecules are dissociated. As indicated above, however, equal volumes of normal hydrochloric and normal acetic acids will be neutralized by exactly the same amounts of base. In other words the titratable acidities of these acids, or the amounts of hydrogen ions which they are capable of yielding on complete dissociation, are the same. The preceding discussion of the hydrogen-ion concentrations and titratable acidities of weak and strong acids applies just as well to the hydroxyl-ion concentrations and the titratable alkalinities of weak and strong bases.

Chemical Equilibrium: Ionization Constants. According to the law of mass action a reversible reaction of the type

$$A + B \rightleftarrows C + D$$

proceeds from left to right at a velocity (v_1) which is proportional to the product of the concentrations of A and B—i.e., $v_1 = k_1 [A] [B]$. Similarly, the opposite reaction takes place at a rate (v_2) proportional to the product of the concentrations of C and D—i.e., $v_2 = k_2 [C] [D]$. At equilibrium the velocities in opposing directions are equal, and hence $k_1 [A] [B] = k_2 [C] [D]$ or $\frac{[C] [D]}{[A] [B]} = \frac{k_1}{k_2} = K$. This expression of the law of mass action states that for a reversible reaction the ratio of the product of the concentrations of the reacting substances on one side of the equation to that of the product of the concentrations of the substances on the other side is constant. That is,

$$\frac{[C] [D]}{[A] [B]} = K$$

Applying this to weak acids, HA, which ionize according to the equation, $HA \rightleftarrows H^+ + A^-$, we find that at equilibrium

$$\frac{[H^+]\,[A^-]}{[HA]} = K_A$$

In this case the equilibrium constant, since it measures the equilibrium between the undissociated molecules of acid and its ions, is known as the dissociation constant, K_A. For weak bases, similarly, the dissociation constant of the base BOH is given by

$$\frac{[B^+]\,[OH^-]}{[BOH]} = K_B$$

From the above equations it is readily seen that the larger the numerical value of the dissociation constants, the more completely the acid or base is dissociated—i.e., the stronger the acid or base.

An acid solution is one that contains an excess of hydrogen ions. An alkaline solution is one that contains an excess of hydroxyl ions. A neutral solution is one that contains hydrogen and hydroxyl ions in equal concentrations. Pure water is a neutral solution. It is dissociated to an extremely small extent according to the equation

$$H_2O \rightleftarrows H^+ + OH^-$$

Applying the law of mass action to this dissociation we get

$$\frac{[H^+]\,[OH^-]}{[H_2O]} = K$$

Since the concentrations of H^+ and OH^- in pure water or in dilute aqueous solutions are so small compared with the concentration of undissociated water molecules, the concentration of the latter, $[H_2O]$, may be considered as constant, and the equation then becomes $[H^+] \times [OH^-] = K\,[H_2O] = K_W$. In other words, the *product* of the concentrations of the H and OH ions is constant. The value of K_W at 25° C. has been found to be 1×10^{-14}. That is, $[H^+] \times [OH^-] = 1 \times 10^{-14}$. In pure water, for every hydrogen ion set free a hydroxyl ion must also be liberated so that the concentrations of the two ions remain equal—that is, $[H^+] = [OH^-]$. Therefore, $[H^+] \times [OH^-] = [H^+]^2 = [OH^-]^2 = 1 \times 10^{-14}$. Then $[H^+] = [OH^-] = \sqrt{1 \times 10^{-14}} = 1 \times 10^{-7}$. Thus pure water or a neutral solution contains approximately 1×10^{-7} moles[22a] of H or OH ions per liter and is a 1/10,000,000 normal solution of H or OH ions.

Hydrogen-ion concentrations are now generally expressed, for practical as well as theoretical reasons, as their logarithms with the sign reversed and indicated by the term pH. Thus[23] $pH = -\log [H^+]$; or $[H^+] = 10^{-pH}$.

[22a] A mole (or gram ion) of hydrogen is 1 g.; of hydroxyl, 16 + 1 = 17 g.

[23] This definition will be used throughout this book, even though it is not quite exact. For example, HCl in 0.1 N solution is completely ionized, so the solution must have a hydrogen-ion concentration of 0.1 N, or 1×10^{-1} moles per liter (pH 1.00). The value determined experimentally by the use of the hydrogen electrode is 83 per cent of this, or 0.83×10^{-1} moles per liter (pH 1.08). Thus if 0.1 N HCl has a pH of 1.08 (a generally accepted value), the pH does not correspond exactly to the negative logarithm of the hydrogen-ion concentration known to be present. This discrepancy becomes less with more dilute solutions.

The logarithm of 1×10^{-7} is -7.0 and the pH is 7.0. To take another example, the hydrogen-ion concentration of 0.1 N HCl is 8.3×10^{-2}. The logarithm of the product of two numbers is equal to the sum of their respective logarithms. In this case, then, $\log (8.3 \times 10^{-2}) = \log 8.3 + \log 10^{-2}$ (consulting table of logarithms[24]) $= 0.92 + (-2) = -1.08$. The pH, being the negative log of the hydrogen-ion concentration, equals $-(-1.08)$ or 1.08. To convert pH values to hydrogen-ion concentrations the procedure is reversed—e.g., in the case of pH 1.08, the $[H^+] = 10^{-1.08} = 10^{-2+0.92} = 10^{-2} \times 10^{0.92}$. The logarithm of a number is the power to which 10 must be raised to give the number, hence the value of $10^{0.92}$ is obtained by looking up the number whose logarithm is 0.92 (i.e., antilog 0.92). This is found to be 8.3. pH 1.08 is therefore equal to a $[H^+]$ of 8.3×10^{-2}.

Since the product of the H and OH ion concentrations is constant at 1×10^{-14}, when the $[H^+]$ increases from 1×10^{-7} (pH 7.0) to 1×10^{-4} (pH 4.0) the $[OH^-]$ decreases to 1×10^{-10} (pOH 10.0). The sum pH + pOH always equals 14. According to this nomenclature an *increase* in pH indicates a *decrease* in hydrogen-ion concentration or true acidity.

Since the hydrogen-ion concentration varies in a definite reciprocal manner with hydroxyl-ion concentration, the pH scale is universally used to express degrees of alkalinity as well as of acidity, as indicated in the second column below.

Normality of $[H^+]$	pH		pOH	*Normality of* $[OH^-]$
N/10	1	Acidity	13	
N/1,000	3		11	
N/1,000,000	6		8	
N/10,000,000	7	Neutrality	7	N/10,000,000
	8	Alkalinity	6	N/1,000,000
	11		3	N/1,000
	13		1	N/10

Buffer Action and Buffers. By buffer action is meant the ability of a solution to resist marked change in pH on the addition or loss of acid or base. Substances whose presence in the solution are responsible for buffer action are known as buffers. Though strong acids or bases may sometimes act as buffers, under ordinary conditions the most common basis for buffer action lies in the presence within the solution of a weak acid (or weak base) together with its salt. Such solutions have characteristic properties with respect to hydrogen-ion concentration which are of value not only in the laboratory but also in the living organism, and an understanding of these properties is of fundamental importance.

If we take for example a solution containing a weak acid, HA, and its salt, BA, the hydrogen ions in the solution can come only from the dissociation of the acid molecules. The dissociation equilibrium of the acid is given by the equation $\frac{[H^+][A^-]}{[HA]} = K_A$, as described previously. From this it follows that the hydrogen-ion concentration $[H^+] = K_A \frac{[HA]}{[A^-]}$. For a weak acid, which is relatively little ionized, the difference between the concentration of undissociated acid molecules [HA] and the total acid concentration [Acid] is small. This difference becomes even less in the presence of a salt of the acid, since the salt ions A^-, by a mass action effect, repress still further the dissociation of acid molecules. Thus in a mixture of salt and acid we can substitute the total acid concentration [Acid] for the quantity [HA], with very little error. Similarly the concentration of acid ions A^- derived from the dissociation of the acid molecules is very small compared to that furnished by the salt BA which is completely dissociated into B^+ and A^- ions, so that we may consider the value of $[A^-]$ to be determined primarily by the salt concentration. Although the salt is completely dissociated, because of the existence of interionic forces the effective concentration of salt ions, $[A^-]$, is somewhat less than the actual salt concentration. The ratio between these two quantities is, however, sensibly constant over the range of concentration that ordinarily comes into question, so that we may say $[A^-]$ equals k[Salt]. Substituting k[Salt] for $[A^-]$ in the above equation, and combining constants, we obtain the expression

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[24] See Appendix.

$$[H^+] = K'_A \frac{[\text{Acid}]}{[\text{Salt}]}$$

This equation tells us that the hydrogen-ion concentration of a solution containing a weak acid and its salt is determined by the value of K'_A for the acid and by the ratio of acid concentration to salt concentration in the solution. While usable in the form given, it is more convenient to convert it into terms of pH rather than of $[H^+]$. If the logarithm of both sides of the equation is taken, and the signs reversed, we obtain:

$$-\log [H^+] = -\log K'_A - \log \frac{[\text{Acid}]}{[\text{Salt}]}$$

Now $-\log [H^+]$ has already been defined as equal to pH. In an analogous way and for similar reasons we can define a term pK'_A as equal to $-\log K'_A$, and we can replace the negative expression $-\log \frac{[\text{Acid}]}{[\text{Salt}]}$ by its equivalent, the positive expression $\log \frac{[\text{Salt}]}{[\text{Acid}]}$. Substituting these various terms in the above equation, we obtain:

$$\text{pH} = pK'_A + \log \frac{[\text{Salt}]}{[\text{Acid}]}$$

This is known as the Henderson-Hasselbalch equation, and is most generally useful. It tells us that the pH of a solution containing a weak acid and its salt is determined by a value pK'_A, which is constant for a particular acid, and by the logarithm of the ratio of salt concentration to acid concentration in the solution. For example, the value of K'_A for acetic acid is approximately 1.8×10^{-5}, or $10^{-4.7}$. The pK'_A for acetic acid is therefore 4.7, and the pH of an acetic-acid–sodium-acetate buffer is given by the equation:

$$\text{pH} = 4.7 + \log \frac{[\text{Sodium acetate}]}{[\text{Acetic acid}]}$$

When [Sodium acetate] = [Acetic acid], the pH is 4.7, since the ratio of salt to acid is 1, and log 1 = 0. By varying the proportion of salt to acid in the mixture the pH will vary over a range of approximately 1.5 pH units below and above the pK'_A value—i.e., from pH 3.2 to pH 6.2. Outside of this range the equation is not applicable for this particular system. To cover other pH ranges, other weak acids with suitable pK'_A values are selected, so that by this means it is possible to prepare buffer solutions of almost any desired pH. This procedure is illustrated in the section on the preparation of pH standards which follows.

In addition to their value in the preparation of solutions of known pH, buffer solutions possess another property of fundamental importance in the living organism; namely, the ability to resist marked changes in pH on the addition of small amounts of strong acids or bases. It has been pointed out by Van Slyke that if a small amount of strong acid such as HCl is added to blood, which contains several buffers, the resulting change in pH in the direction of increased acidity may be only 1/1,000 of that which would have occurred if the blood were unbuffered. The action of a buffer solution in minimizing the effect on pH of added acid or base may be visualized as follows.

When base is added to the mixture the excess hydroxyl ions are removed by the hydrogen ions coming from the acid, which combine with them to form water. Upon the addition of acid the excess hydrogen ions from the added acid are removed by the salt ions, which combine with them to form more molecules of the relatively un-ionized weak acid. The reactions involved are as follows:

$$\begin{array}{l} \text{Buffer} \\ \text{Mixture} \end{array} \left\{ \begin{array}{l} \text{Acid: HA} \rightleftarrows \text{H}^+ + \text{A}^-\text{; on addition of base, OH}^- + \text{H}^+ \rightarrow \text{H}_2\text{O} \\ \text{Salt: BA} \rightleftarrows \text{B}^+ + \text{A}^-\text{; on addition of acid, H}^+ + \text{A}^- \rightarrow \text{HA} \end{array} \right.$$

Thus the addition to a buffer solution of small amounts of base results merely in the production of more salt ions at the expense of an equivalent amount of the weak acid present; the addition of acid similarly results in the production of more weak acid at the expense of the salt. *The pH of the solution must inevitably change,* but this change in accordance with the demands of the Henderson-Hasselbalch equation is due to a change in the value of the logarithm of the ratio of salt concentration to acid concentration, which numerically is quite small in comparison to the concentration of H^+ or OH^- ions added to the solution.

It is obvious from what has just been said that the more concentrated a buffer is, the smaller will be the change in pH on the addition of a given amount of strong acid or base—i.e., the greater will be the buffer power of the solution. Furthermore, if for example acid is added to a buffer solu-

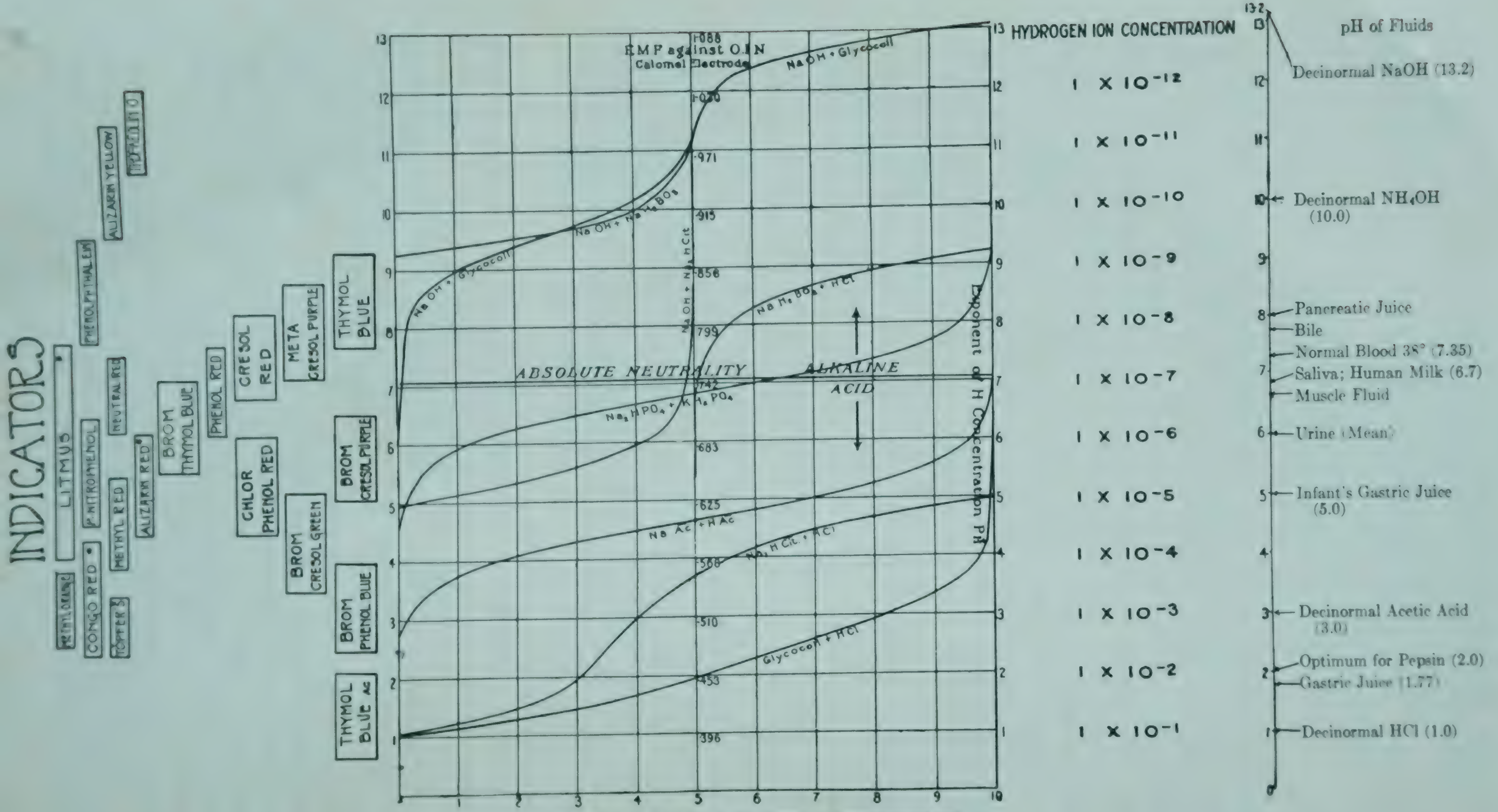

FIG. 6. HYDROGEN-ION CONCENTRATION CHART.

This chart is, in slightly modified form, one prepared by Walpole from a large amount of data accumulated by Sørensen, Walpole himself, Michaelis, and a number of other authors. See Walpole: *Biochem. J.*, 8, 628 (1914). Indicators marked * are used in titrations but not for pH tion in an amount greater than that equivalent to the buffer salt present, the buffer capacity of the system will be exceeded and its ability to function as a buffer will disappear, since it will no longer consist of a mixture of buffer salt and acid. Another property of buffer solutions, which is also evident from inspection of the Henderson-Hasselbalch equation, is that they may be diluted considerably without appreciable change in pH, since the pH depends upon a concentration ratio rather than upon concentration itself.

The above discussion has been based entirely upon a consideration of buffers containing weak acids and their salts. An entirely analogous derivation can be made for solutions containing weak bases and their salts.

Preparation of Standard Buffers of Known pH. Fig. 6 indicates how standard solutions of definite hydrogen-ion concentration may be made up from a series of stock solutions, mixed in definite proportions. The stock solutions indicated on the chart were suggested by Sørensen and are as follows: 0.10 N HCl; 0.10 N NaOH; 7.505 g. glycine plus 5.85 g. NaCl per liter; 11.876 g. $Na_2HPO_4 \cdot 2H_2O$ per liter;[25] 9.078 g. KH_2PO_4 per liter; 21.008 g. citric acid in 1 liter of 0.20 N NaOH; 12.404 g. boric acid in 1 liter of 0.10 N NaOH. The other solutions are 0.20 N sodium acetate and 0.20 N acetic acid. Solutions of known hydrogen-ion concentration are prepared from these by mixing in the proportions indicated in Fig. 6, the abscissas representing parts of the relatively more alkaline constituent. Thus a mixture of seven volumes of the sodium acetate stock solution with three volumes of the stock acetic acid solution gives a mixture with a hydrogen-ion concentration of 1×10^{-5} (pH 5.0). The mixtures are most satisfactory through the ranges where the hydrogen-ion concentrations change most gradually—that is, through the flatter portions of the curves. The phosphate mixtures covering the range 5.3 to 8.0 are especially useful in biological work. The standard buffer solutions of Clark and Lubs, described below, are arranged to differ by even increments in pH instead of in component solutions, and are quite generally used.

BUFFER SOLUTIONS OF CLARK AND LUBS:[26] *Group 1.* To 50 ml. of

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[25] This sodium phosphate may be obtained from laboratory supply houses or may be prepared by drying the powdered crystals containing 12 molecules of water by exposure to air at room temperature in shallow dishes for two weeks or more. To further insure dryness portions of the salt may then be placed in an incubator at 36° to 38° C. for one or two days.

The water used in preparing these solutions should be distilled water that has been boiled in a pyrex flask to eliminate CO_2. Stopper the flask while still hot with a rubber stopper carrying a soda-lime tube, and allow to cool. The sodium hydroxide must be carbonate-free (see Clark and Lubs' solutions). Protect the solutions from the CO_2 of the air.

[26] The constituent solutions are prepared as follows:

0.2 M *potassium chloride.* Dissolve 14.912 g. in distilled water and make up to 1 liter. The salt should be recrystallized and dried at about 120° C. for two days.

0.2 M *acid potassium phthalate.* Dissolve 40.828 g. in distilled water and make up to 1 liter. The salt should be recrystallized from distilled water and dried at 110° to 115° C. for some hours.

0.2 M *acid potassium phosphate.* Dissolve 27.232 g. in distilled water and make up to 1 liter. The salt should be recrystallized from distilled water and dried at 110° to 115° C. for some hours.

0.2 M *boric acid in* 0.2 M *potassium chloride.* Dissolve 12.4048 g. of air-dried boric acid and 14.912 g. pure KCl in distilled water and make up to 1 liter.

0.2 N *sodium hydroxide.* Dissolve 100 g. of the best NaOH in 100 ml. of distilled water in an Erlenmeyer flask (pyrex). Cover the mouth of the flask with tinfoil, and allow the

0.2 M KCl add the indicated number of ml. of 0.2 N HCl and dilute to 200 ml. Indicator: thymol blue.

pH	HCl	pH	HCl	pH	HCl	pH	HCl
1.2	64.5	1.6	26.3	2.0	10.6	2.2	6.7
1.4	41.5	1.8	16.6	..	..	..	..

Group 2. To 50 ml. of 0.2 M acid potassium phthalate add the indicated number of ml. of 0.2 N HCl and dilute to 200 ml. Indicators: thymol blue and brom phenol blue.

pH	HCl	pH	HCl	pH	HCl	pH	HCl
2.2	46.70	2.8	26.42	3.2	14.70	3.6	5.97
2.4	39.60	3.0	20.32	3.4	9.90	3.8	2.63
2.6	32.95	..	..	..	..	..	..

Group 3. To 50 ml. of 0.2 M acid potassium phthalate add the indicated number of ml. of 0.2 N NaOH and dilute to 200 ml. Indicators: brom phenol blue, brom cresol green, and brom cresol purple.

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
4.0	0.40	4.6	12.15	5.2	29.95	5.8	43.00
4.2	3.70	4.8	17.70	5.4	35.45	6.0	45.45
4.4	7.50	5.0	23.85	5.6	39.85	6.2	47.00

Group 4. To 50 ml. of 0.2 M acid potassium phosphate add the indicated number of ml. of 0.2 N NaOH and dilute to 200 ml. Indicators: brom cresol purple, brom thymol blue, and phenol red.

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solution to stand overnight till the carbonate has settled. Cut a hardened filter paper to fit a Buchner funnel. Treat it with warm, strong (1:1) NaOH solution. Decant the soda and wash the paper first with absolute alcohol, then with dilute alcohol, and finally with large quantities of distilled water. Place the paper on the Buchner funnel and apply gentle suction until the greater part of the water has evaporated. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod, and filter under suction. The clear solution is now diluted quickly with cold distilled water that has been boiled recently to remove CO_2, to make approximately N NaOH (about 50 ml. per liter). Ten ml. of this are withdrawn and roughly standardized against N HCl. It is then diluted till it is approximately 0.2 N with CO_2-free water and the solution poured into a paraffined bottle, to which a buret and soda-lime guard tubes have been attached. The solution is then accurately standardized against weighed amounts of the pure acid potassium phthalate. To do this, weigh accurately about 1.6 g. of the salt, dissolve in about 30 ml. of distilled water, add phenolphthalein, and titrate with the alkali till a faint but distinct and permanent pink is developed. A current of CO_2-free air should be blown through the solution during the titration.

$$\frac{\text{g. of phthalate used} \times 1000}{204.14 \times \text{ml. NaOH required}} = \text{Normality of NaOH}$$

It is not necessary to make exactly 0.2 N, but the true strength must be considered in making standards.

0.2 N *hydrochloric acid.* Dilute concentrated HCl to 20 per cent. Distil, dilute the distillate, and standardize against the standard soda, using methyl red as the indicator. (Clark: *Determination of Hydrogen Ions*, 3d ed., Baltimore, Williams and Wilkins Co., 1928.)

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
5.8	3.72	6.4	12.60	7.0	29.63	7.6	42.80
6.0	5.70	6.6	17.80	7.2	35.00	7.8	45.20
6.2	8.60	6.8	23.65	7.4	39.50	8.0	46.80

Group 5. To 50 ml. of 0.2 M boric acid in 0.2 M KCl add the indicated number of ml. of 0.2 N NaOH and dilute to 200 ml. Indicators: cresol red and thymol blue.

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
7.8	2.61	8.4	8.50	9.0	21.30	9.6	36.85
8.0	3.97	8.6	12.00	9.2	26.70	9.8	40.80
8.2	5.90	8.8	16.30	9.4	32.00	10.0	43.90

Theory of Indicators. Indicators may usually be regarded as weak organic acids (or bases) whose un-ionized molecules exhibit one color whereas their anions (or cations) possess a different color. In the case of an indicator which behaves as a weak acid, for example, we have the following equilibrium reaction:

$$\underset{\text{(Color A)}}{\text{H Indicator}} \rightleftarrows \text{H}^+ + \underset{\text{(Color B)}}{\text{Indicator}^-}$$

Applying the law of mass action to this ionization of the indicator acid, we get

$$\frac{[\text{H}^+]\,[\text{Indicator}^-]}{[\text{H Indicator}]} = K', \text{ or } \frac{[\text{H}^+]}{K'} = \frac{[\text{H Indicator}]}{[\text{Indicator}^-]}$$

where K' is called the apparent dissociation constant of the indicator.

The color imparted to a solution by the indicator depends upon the relative proportions of the two forms of the indicator present in the solution, and this, in turn, depends upon the ratio $[\text{H}^+]/K'$. Since K' is a fixed number, specific for each indicator, the color formed will depend upon the hydrogen-ion concentration of the solution. In the case of brom cresol green, for example, $K' = 10^{-4.7}$; the undissociated molecules are yellow while the anions are blue. For this indicator, therefore, the above equation may be written

$$\frac{[\text{H}^+]}{10^{-4.7}} = \frac{[\text{Yellow molecules}]}{[\text{Blue ions}]}$$

If brom cresol green is added to a buffer solution whose hydrogen-ion concentration is $10^{-4.7}$ (i.e., is numerically equal to the value of K' for the indicator), then according to the above equation the concentrations of yellow molecules and blue ions in the solution must be equal. In this case therefore the solution will assume the green color which results when equal numbers of these yellow and blue particles are mixed together. If, on the other hand, the indicator is added to a series of solutions whose hydrogen-ion concentrations are progressively greater than $10^{-4.7}$, then, as the hydrogen-ion concentrations increase, the proportion of yellow molecules to blue ions in these mixtures will also increase. Finally, at a hydrogen-ion concentration of about $10^{-3.8}$ (pH = 3.8), the eye can no longer detect the presence of blue ions in the mixture. At this pH the solution exhibits the yellow color of the undissociated indicator molecules, and further increases in hydrogen-ion concentration will not produce any perceptible changes in color. Similarly in solutions whose hydrogen-ion concentrations are less than $10^{-4.7}$ the blue anions predominate, and as the hydrogen-ion concentration decreases a point is reached where it is the yellow molecules which no longer can be detected in the mixture. At this pH (approximately 5.4), the solution exhibits the blue color of the indicator ions, and further reduction in hydrogen-ion concentration produces no perceptible change in the color. Thus in solutions having a pH between 3.8 and 5.4 the indicator exists as a mixture of yellow molecules and blue ions and the relative amounts of each of these substances in the mixture, and the colors formed, vary with the hydrogen-ion concentrations of the solutions. In this range therefore brom cresol green can be used to determine the pH of an unknown solution by comparing the color formed with the colors produced when the indicator is added to a series of standard buffer solutions of known pH. In solutions whose pH is less than 3.8 or more than 5.4, however, the eye can detect the presence of only one form of the indicator and variations in pH are no longer accompanied by visible changes in color. The pH of such solutions, therefore, cannot be determined by means of this particular indicator. Since each indicator has its own value for K' it follows that for each indicator there is a definite range of hydrogen-ion concentration in which the indicator exists as a mixture of the ionized and un-ionized forms, and in which variations in hydrogen-ion concentration will be accompanied by visible changes in color. The problem of determining the pH of an unknown solution by means of indicators depends, therefore, upon the selection of an indicator whose effective range includes the pH of that particular solution.

Uses of Indicators. Both the concentration of free hydrogen ions in a solution and its titratable acidity (or alkalinity)—i.e., the total amount of hydrogen (or hydroxyl) ions which the solution is capable of yielding on complete dissociation—may be determined by the use of proper indicators. For the latter determination the solution is titrated with standard alkali or acid in the presence of an indicator which serves as an index of the end point of the reaction. The indicator used for this purpose should be one which gives a sharp color change when an equivalent amount of the standard acid or alkali has been added. If at this point the reaction of the solution is practically neutral (pH 7), an indicator changing color at about this reaction (litmus or rosolic acid) would be suitable. On the other hand if we add 20 ml. of 0.1 N NaOH to 20 ml. of 0.1 N acetic acid the resulting solution will be not neutral, but rather slightly alkaline due to hydrolysis. The sodium acetate formed will react with the water to produce some NaOH and an equivalent amount of acetic acid. Acetic acid is a weak acid and will dissociate few hydrogen ions, but NaOH is a strong base and dissociates completely, liberating OH ions which, being in excess, cause the solution to be alkaline. In titrating weak acids such as acetic and most other organic acids it is therefore necessary to use an indicator which changes color in a slightly alkaline medium. Phenolphthalein has been found to be suitable. For similar reasons the titration of weak bases such as ammonia necessitates the use of an indicator which changes color in an acid medium. Methyl red and alizarin red are often used in such cases. If a strong base such as NaOH is titrated with a strong acid such as HCl, almost any common indicator may be used, because one drop of 0.1 N solutions of these will throw the hydrogen-ion concentration so far beyond that of neutrality as to pass the turning point of any of these indicators. (For further discussion see electrometric titration method, p. 50, and titration curves, Fig. 11, p. 50.)

In making use of indicators for determination of hydrogen-ion concentration, the first problem is to choose an indicator whose effective range includes the pH of the unknown solution. That is, the indicator used must be one which, when added to the unknown solution, will exist as a mixture of both the ionized and un-ionized forms, so that the color will be characteristic of the pH. After selection of the proper indicator the unknown is treated with a measured volume of this indicator and the color obtained is compared with those produced when the same amount of indicator is added to a series of buffer solutions of known hydrogen-ion concentration. The pH of the unknown is considered to be the same as that of the buffer solution which yields exactly the same shade of color. For use in the determination of the hydrogen-ion concentration of a solution an indicator is selected which shows a satisfactory gradation in color through the range in question, which is not readily affected by the presence of neutral salts or other substances likely to be present, and the color of which does not fade too rapidly. The ranges through which a number of indicators may be used with satisfactory results for the determination of hydrogen-ion concentrations are indicated in Fig. 6. The sulfonphthalein series of Clark and Lubs is especially brilliant and reliable.

EXPERIMENTS ON DETERMINATION OF HYDROGEN-ION CONCENTRATION

1. Colorimetric Determination of Hydrogen-ion Concentration. **The first step in this procedure is to determine the approximate pH of the unknown solution so that a suitable indicator may be selected. Treat a small (about a 1-ml.) portion of the unknown solution with 1 drop of an indicator[27] solution,**

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[27] Indicators may be obtained in powder form or in prepared solutions from LaMotte Chemical Products Co., W. A. Taylor and Co., or Hynson, Westcott, and Dunning, all of Baltimore, Md. 0.04 per cent solutions of all of the Clark-Lubs series of indicators are used. For many purposes 0.04 per cent solutions of the indicators in 95 per cent alcohol may be used. Clark and Lubs prefer to use aqueous solutions of the alkali salts. To prepare these stock solutions rub 0.1-g. portions of dry powder in an agate mortar with the following quantities of 0.05 N NaOH: phenol red 5.7 ml., brom phenol blue 3.0 ml. cresol red or meta cresol purple 5.3 ml., thymol blue 4.3 ml., brom thymol blue 3.2 ml., brom cresol green 2.9 ml., chlor phenol red 4.7 ml. If exact equivalents are not desired, solution is more readily obtained by using 1.1 equivalents of NaOH. When solution is complete, dilute to 25 ml. with water. Dilute portions of these stock solutions ten times to get 0.04 per cent solutions. Other indicator solutions may be made up as follows: Alizarin yellow R (*p*-nitrobenzene-azo-salicylic acid), methyl orange, tropaeolin O, tropaeolin OO, and tropaeolin OOO in 0.01 per

and compare the color obtained with those resulting when 1 drop of the indicator is added to the same volume of 0.1 N HCl (acid color) and 0.1 N NaOH (alkaline color). If the color obtained with the unknown is intermediate between the acid and alkaline colors of the indicator, the pH of the unknown lies within the effective range of this indicator and it may be used for the colorimetric determination of pH as described below. If on the other hand the unknown shows either the full acid or full alkaline color with the indicator selected, it is unsuitable and another indicator must be tried in a similar manner, until an indicator has been found whose effective range includes the pH of the unknown solution.

When the proper indicator has been selected, look up the effective pH range of this indicator (see chart, p. 34, or table, p. 381). Into a series of clean test tubes of clear (preferably pyrex) glass of uniform internal diameter (about 15 mm.), measure 10-ml. portions of standard buffer solutions covering this range, preferably in steps of 0.2 pH unit. Add 10 drops (0.5 ml.) of indicator solution to each tube and mix. Prepare a 10-ml. portion of the unknown solution in a test tube similar to that containing the standards and with the same amount of added indicator. Compare the unknown against the standards, using a comparator block (Fig. 7). The pH of the standard most closely matching the unknown is the pH of the unknown. It should be possible to interpolate between standards, thus attaining a precision of 0.1 pH unit, if the standards differ by 0.2 pH.

For approximate orientation in the preliminary trials, suitable use may be made of the ordinary laboratory indicators (litmus, phenolphthalein, methyl orange—see Fig. 6), but the final choice of indicator should be based upon trial with those indicators which have been specially selected for use in the colorimetric determination of hydrogen-ion concentration. In this connection it is well to remember that thymol blue has two effective pH ranges (1.2 to 2.8, and 8.2 to 9.8), the alkaline color for the first range as well as the acid color for the second range being obtained with some solution having a pH between 3 and 8, such as dilute acid potassium phosphate solution. In measuring out the indicators the dropping pipet should be held perpendicularly so that drops of constant size are obtained. In measuring out buffer solutions with a pipet the latter should not be blown out and if used for another solution must first be rinsed very thoroughly with distilled water from a flask which has not been blown through (because of the CO_2 in expired air).[28] Color comparisons should be made in a good light using a sheet of white paper as a background.

If the stock solutions of Sørensen are used, 10-ml. portions of standard solutions may be made up from these as required. The stock solutions are measured from burets and the proportions required for a certain pH may be found in Fig. 6. Let us suppose that the unknown solution has been found to have a pH within the range of phenol red (6.6 to 8.2). This range is covered by the phosphate buffer mixtures. Measure out 5 ml. of the acid phosphate and 5 ml. of the alkaline phosphate, mix, and add 10 drops of indicator. If the same tint is obtained as with the unknown, consult the chart. It will be found that this phosphate mixture has a pH of 6.8 and the pH of the unknown must also be 6.8. If the same tint is not obtained on the first attempt make other trials

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cent aqueous solutions. Methyl red, saturated solution in 50 per cent alcohol. Neutral red, 0.01 per cent in 50 per cent alcohol. *p*-Nitrophenol, 0.04 per cent in 5 per cent alcohol. Phenolphthalein, 0.5 per cent in 50 per cent alcohol. Azolitmin in aqueous solution.

[28] For the standards it is best to use bottles whose stoppers carry 10-ml. pipets. Where used by more than one person, bulbs may be used to fill the pipets.

using proportions of 4:6, 3:7, etc. For other ranges of pH use other indicators and other buffer solutions in the same manner.

If the unknown solution possesses a color of its own either of two procedures may be resorted to. The first method is to add to the standard, before any indicator is added, enough inert coloring matter to match that in the unknown and then add indicator to both.[29] A better method which may also be applied to moderately turbid solutions was suggested by Walpole. The comparator shown in Fig. 7 is used.[30] The colored fluid plus indicator is placed in a test tube in hole 3 and the standard solutions for comparison on each side in holes 1 and 5. Then opposite the standards in holes 2 and 6 are placed tubes containing the colored unknown solution without added indicator and in hole 4 a tube containing water only. Comparison is made through the apertures A, B, and C.

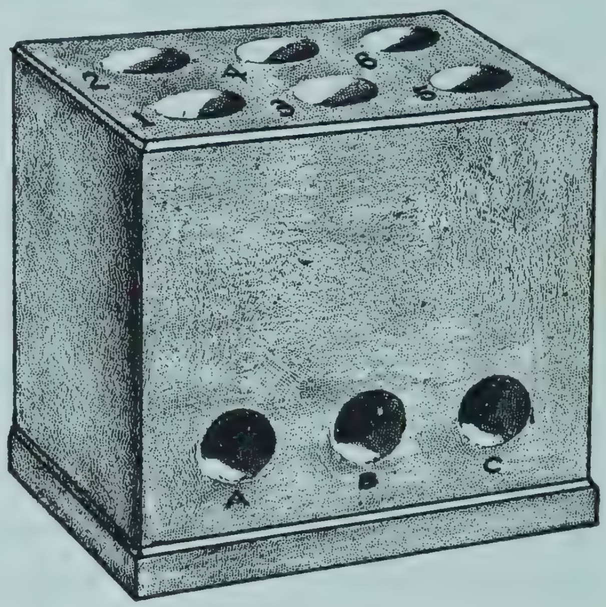

FIG. 7. COMPARATOR BLOCK.

2. *Colorimetric Determination of Hydrogen-ion Concentration Without Use of Buffer Solutions.*[31] This procedure is most simply carried out using indicators changing from colorless to a single colored form such as phenolphthalein or the nitrophenols. Within the effective range of the indicator, the depth of color varies with the pH and depends upon the amount of dissociated indicator present. The amount of completely dissociated indicator required to give the same tint as the unknown is a measure of the pH of the latter. The standards are made up by adding measured amounts of indicator solution to portions of solution in which the indicator gives its maximum color (complete dissociation).

To 10 ml. of unknown solution in a test tube add 1 ml. of indicator solution.[32] To another tube (the standard) add 9 ml. of a solution giving the

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[29] Solutions suitable for this purpose include Bismarck brown, tropaeolin O and tropaeolin OO in 0.02 per cent aqueous solutions, cotton blue 0.01 per cent, methyl violet 0.002 per cent, methyl orange 0.01 per cent, all in water; curcumine 0.02 per cent in 60 per cent alcohol, and helianthin II 0.01 per cent in 80 per cent alcohol.

[30] A comparator may be made by boring 6 holes in pairs in a block of soft wood, the paired holes being as close together as possible and just large enough to hold ordinary test tubes. *Smaller* holes are then bored perpendicular to these. Stain black with an alcohol wood stain. A sheet of ground glass placed between the light source and the tubes is helpful.

[31] Michaelis and Gyemant: *Biochem. Z.*, **109**, 165 (1920).

[32] Michaelis suggests the following indicators:

Name	*Composition*	*pK′ 18° C.*	*Range of pH*	*Stock Solution of Indicator*
β-Dinitrophenol.......	1-oxy-2, 6-dinitrobenzol	3.69	2.2–4.0	0.1 g.: 300 ml. H_2O
α-Dinitrophenol.......	1-oxy-2, 4-dinitrobenzol	4.06	2.8–4.5	0.1 g.: 200 ml. H_2O
γ-Dinitrophenol.......	1-oxy-2, 5-dinitrobenzol	5.15	4.0–5.5	0.1 g.: 200 ml. H_2O
p-Nitrophenol........	..	7.18	5.2–7.0	0.1 g.: 100 ml. H_2O
m-Nitrophenol........	..	8.33	6.7–8.4	0.3 g.: 100 ml. H_2O
Phenolphthalein......	..	9.73	8.5–10.5	0.04 g.: 30 ml. alcohol + 70 ml. H_2O

maximum color with the indicator (e.g., 0.02 N NaOH) and then add indicator solution diluted ten times from a microburet or Mohr pipet until a color is obtained which on diluting the standard to the same volume as the unknown (11.0 ml.) matches that of the unknown. If the first standard does not exactly match, make a second one; if more than 2 ml. of diluted indicator are needed, repeat, using undiluted indicator.

CALCULATION.

$$\text{pH} = \text{p}K' + \log \frac{C}{1 - C}$$

where pK' is a constant for the indicator (consult table) and C is the volume in ml. of indicator added to the standard, expressed on an undiluted basis. If 1 ml. of diluted indicator were used (0.1 ml. of undiluted) and the indicator is *p*-nitrophenol (pK' 7.18) we have $\text{pH} = 7.18 + \log \frac{0.1}{0.9} = 7.18 + (\log 0.1 - \log 0.9) = 7.18 + (-1 - (9.95 - 10)) = 7.18 - 0.95 = 6.23$. A chart giving values of $\log \frac{C}{1 - C}$ is convenient in making calculations.[33]

Other Colorimetric Methods for Determining Hydrogen-ion Concentration. The method without buffers may also be applied to two-color indicators (Gillespie). In this case each standard consists of two tubes set one behind the other in the Walpole comparator. One tube contains for example a known amount of phenol red in alkaline solution (red) and the other in acid solution (yellow). By varying the proportions any shade of this indicator may be obtained. For further details, see Clark.

Indicator paper ("Hydrion," "Accutint," etc.) may also be used for the determination of pH. Such paper is convenient and sufficiently accurate for many purposes.

3. *Comparison of Hydrogen-ion Concentration and Titratable Acidity.* (A) Determine colorimetrically the hydrogen-ion concentration of a 0.01 N solution of hydrochloric acid using thymol blue as an indicator and of a 0.01 N acetic acid solution using brom cresol green as an indicator. Note the great difference between the true acidities of the two solutions.

Titrate 10-ml. portions of 0.01 N hydrochloric acid and of 0.01 N acetic acid with 0.01 N NaOH, using phenolphthalein as an indicator. Note that identical results are obtained for the titratable acidities of the two.

(B) Mix equal portions of M/15 potassium dihydrogen phosphate and M/15 disodium phosphate (see chart). Note that the mixture is practically neutral to litmus. Titrate one 10-ml. portion of this mixture with 0.1 N NaOH, using phenolphthalein as an indicator. Titrate another portion with 0.1 N HCl solution, using methyl orange. Explain results.

4. *Differential Titration of Strong and Weak Acids.* Mix 5.0 ml. of 0.1 N HCl with 5.0 ml. of 0.1 N acetic acid. Add thymol blue, and titrate with 0.1 N NaOH to pH 2.8 to 3.0 (use a pH standard as a control). Read the buret, then continue titrating until the indicator changes to blue (pH 8.5). Again read the buret. Compare the buret readings with those expected if (a) only the strong acid were titrated and (b) both acids were completely titrated. Explain. This principle finds clinical application in the titration of gastric juice.

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[33] For chart, see Michaelis: *Practical Physical and Colloid Chemistry*, translated by Parsons, Cambridge, W. Heffer and Sons, 1925; and Clark: *Determination of Hydrogen Ions*, 3d ed., Baltimore, Williams and Wilkins Co., 1928.

ELECTROMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATION

Electrode Potentials. If an excess of solid glucose is placed in water, some of the molecules of the solid, being in a continual state of vibration, escape into the liquid. As the molecules of glucose in solution accumulate, some of them collide with the particles of solid and are retained. An equilibrium is finally established in which there is a balanced exchange of molecules between the solid and the solution. At this stage the solution is saturated with glucose and the solution pressure of the solid is said to be equal to the osmotic pressure of the dissolved substance.

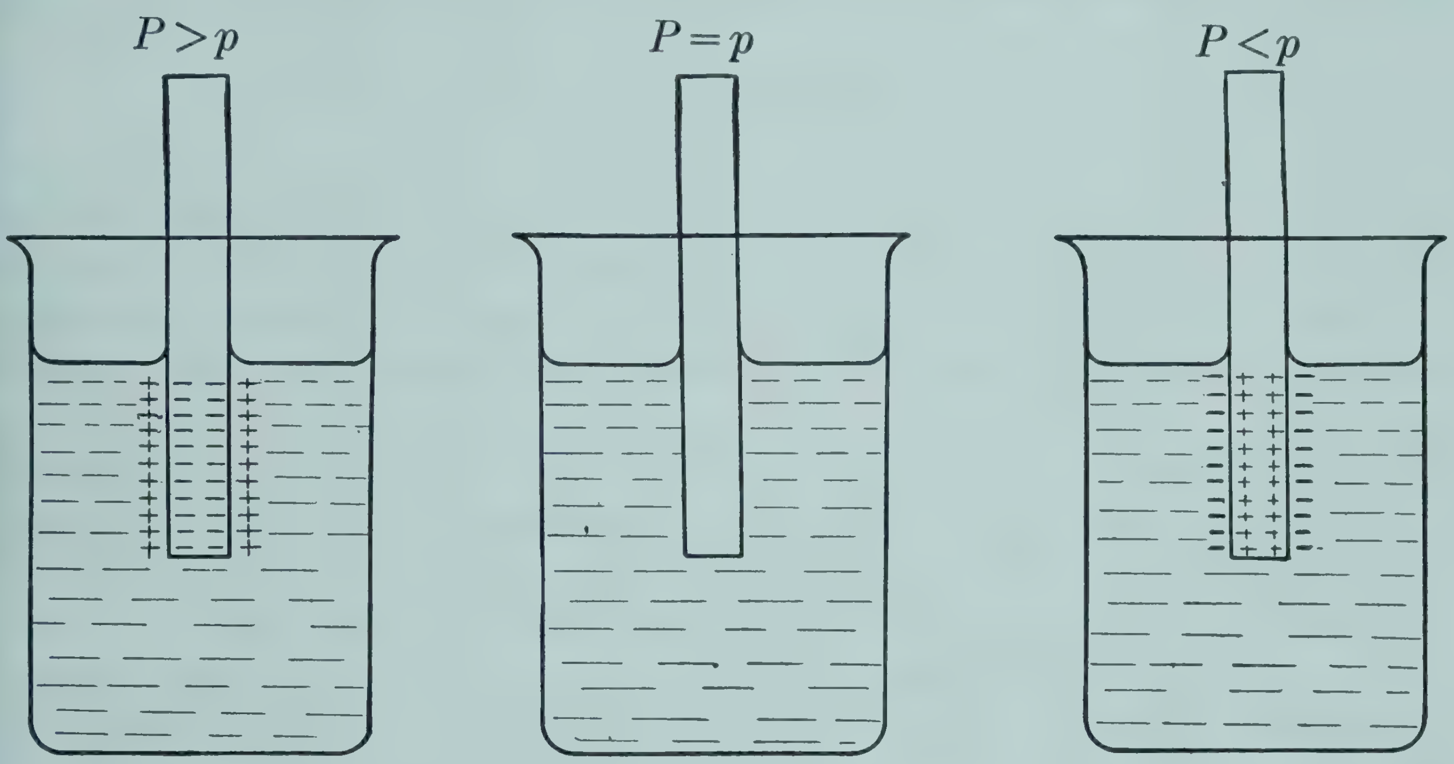

FIG. 8. DIAGRAM OF RELATIONS AT SURFACE OF A METAL DIPPING INTO SOLUTIONS CONTAINING ITS IONS IN DIFFERENT CONCENTRATIONS.
(P = solution pressure of the metal; p = osmotic pressure of metallic ions.)

If a strip of metal (electrode) is dipped into water, it also tends to dissolve owing to its solution pressure, P. The metal goes into solution as positive ions leaving its electrons (presumably less firmly bound valence electrons) behind as a negative charge on the electrode. This charge, by attracting the positive ions of metal already in solution, builds up an electrical double layer at the interface between electrode and solution which opposes the entrance of any more positive ions into the solution, and the process of solution ceases therefore when this charge becomes large enough to prevent the further separation of positive ions from the metal. Although equilibrium is established before a measurable quantity of the metal dissolves, since the charge on each ion is comparatively large, a measurable potential difference is developed between the electrode and the solution.

When a metal is dipped into a solution of one of its salts the conditions are somewhat altered because the positive ions of the metal already in solution oppose the separation of more ions from the metal. The point of equilibrium therefore will depend upon the relative values of the two opposing forces: the solution pressure (P) of the metal and the osmotic pressure (p) of its ions in solution. We may therefore distinguish three possibilities (see Fig. 8):

1. $P > p$. In this case the metal continues to send positive ions into the solution until the accumulated charges oppose further action. The metal thus acquires a negative charge relative to the solution.
2. $P < p$. In this case the positive ions in solution deposit on the metal, yielding to it their positive charges, until the accumulated charges oppose further deposition. The metal thus acquires a positive charge relative to the solution.
3. $P = p$. In this case there is neither solution nor deposition and no potential difference develops between the metal and the solution.

Nernst has shown by thermodynamic reasoning that the potential difference between a metal and a solution of one of its salts is given by

$$E = \frac{RT}{nF} \ln \frac{P}{p}$$

where E = electrode potential, R = gas constant = 8.316 joules per degree, T = absolute temperature, n = valency of metal ion, F = faraday = 96,500 coulombs, P = solution pressure, and p = osmotic pressure of metal ions in solution. (The symbol "ln" stands for the natural logarithm.)

From this equation it becomes evident that, since RT/F is a constant for any given temperature, the magnitude and sign of the electrode potential, E, is determined by n and P, which depend solely upon the nature of the metal used, and p, which is a function of the concentration of metal ions in solution. If therefore we have two electrodes of the same metal dipping into solutions of different concentrations, the two electrode potentials will be different. And if we make suitable connections[34] between the two electrodes and the two solutions an electrical current will pass from one electrode to the other, the electromotive force of which will be equal to the difference between the two electrode potentials—i.e.

$$\begin{aligned} E &= E_1 - E_2 \\ &= \frac{RT}{nF} \ln \frac{P_1}{p_1} - \frac{RT}{nF} \ln \frac{P_2}{p_2} \\ &= \frac{RT}{nF} (\ln P_1 - \ln p_1 - \ln P_2 + \ln p_2) \\ &= \frac{RT}{nF} \ln \frac{p_2}{p_1} \text{ (since } P_1 = P_2 \text{ for the same metal)} \end{aligned}$$

If osmotic pressure is regarded as proportional to concentration, we may replace p_1 and p_2 by c_1 and c_2, and get

$$E = \frac{RT}{nF} \ln \frac{c_2}{c_1}$$

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[34] The two solutions are usually connected by a salt bridge consisting of an inverted U-tube filled with saturated KCl solution. This tends to eliminate any differences of potential which may be built up at the junction of the two solutions by the unequal diffusion of the ions. The electrodes are connected by wire in the ordinary manner.

An arrangement such as here described is known as a concentration cell, and the above equation permits us to calculate the concentration of metal ions in an unknown solution from the electromotive force developed when that solution is combined in a cell with a similar solution of known ionic concentration. It should be noted that the concentration calculated by the use of the above equation will be that of electromotively active ions, which is not necessarily the same as that obtained by ion conductivity or other means. For a further discussion of this point, the reader is referred to Clark.

The Hydrogen Electrode. A strip of platinum, coated with platinum black and saturated with hydrogen gas, acts exactly as the metal electrodes described above when dipped into a solution containing hydrogen ions. We can therefore construct a concentration cell by dipping two such electrodes into two solutions of different hydrogen-ion concentrations, and, if the concentration of hydrogen ions in one solution is kept at a known constant value, we can use the electromotive force developed by such a cell to calculate the hydrogen-ion concentration in the second unknown solution. If the concentration of hydrogen ions in the known solution is normal, the equation for the electromotive force becomes $E = \frac{RT}{nF} \ln \frac{1}{[H^+]}\cdot$ By substituting the values for R, T, n, and F at 25° C. and multiplying by 2.303 to change from the natural to the common system of logarithms, we get $E = 0.059 \log \frac{1}{[H^+]}$ or $E = 0.059\ (-\log [H^+])$. Hydrogen-ion concentrations, following the convention introduced by Sørensen, are usually expressed in terms of pH; by definition, $\text{pH} = -\log [H^+]$. Therefore, $E = 0.059\ \text{pH}$ or $\text{pH} = \frac{E}{0.059}\cdot$

In practice it is convenient to use, instead of a hydrogen electrode in a normal solution of hydrogen ions, a calomel electrode which has been standardized against such a solution. A calomel electrode is made up of mercury in contact with calomel in a solution of potassium chloride, the latter being either normal, tenth-normal, or, more often, saturated. The normal hydrogen electrode is approximately 0.25 volt more positive than the saturated calomel electrode[35] so that the difference of potential between the calomel electrode and the unknown will be 0.25 volt greater than that between the latter and the normal hydrogen electrode. Therefore, using the saturated calomel electrode at a temperature of 25° C., $\text{pH} = \frac{E - 0.246}{0.059}\cdot$ We may read E directly in volts on a voltmeter, or by means of a potentiometer, so that the calculation becomes very simple. Variations of pH with E may be plotted in the form of a curve. In some instruments the voltmeter is graduated to read pH directly.

Regardless of the types of electrodes used in the chain, the measurement of E must be conducted by means which do not entail an appreciable flow of current from the system, because such a flow introduces polarization effects at the electrodes. The potentiometric method is ideally adapted for this purpose. The principles involved in this method are described in detail in Clark's book, and also in the descriptive literature which accompanies the special apparatus required.[36]

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[35] This figure varies with the temperature and with the concentration of KCl solution in the calomel electrode (see values for E_0 in the table on p. 48).

Hildebrand's method, described below, requires only such electric apparatus as is usually found in any laboratory, and serves to illustrate the principles of the electrometric method. The use of a potentiometer instead of the voltmeter indicated increases the accuracy of the determination and is to be preferred.

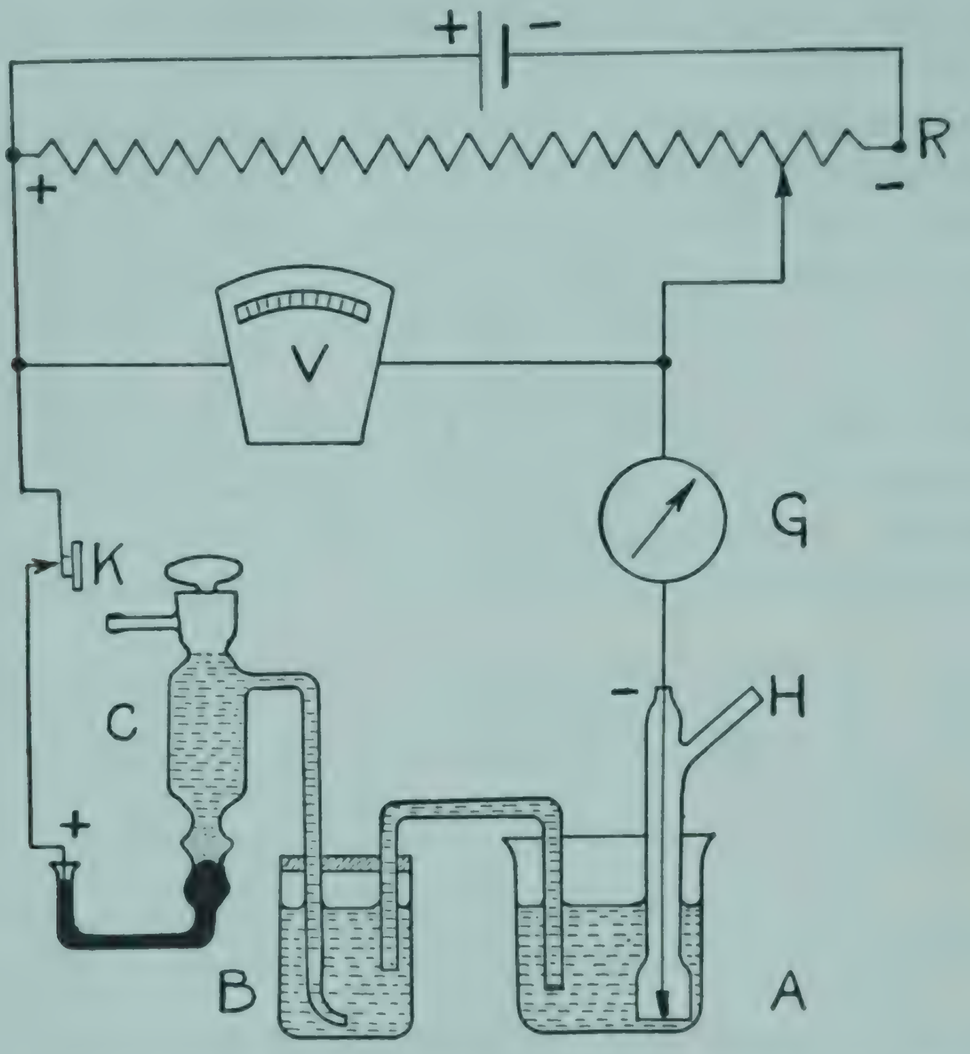

FIG. 9. ARRANGEMENT OF APPARATUS FOR ELECTROMETRIC DETERMINATION OF HYDROGEN-ION CONCENTRATION. (METHOD OF HILDEBRAND.)

H is the hydrogen electrode, *C* the calomel electrode, *V* a voltmeter, *G* a galvanometer, *R* a rheostat, *A* the beaker containing the unknown solution, and *B* a connecting vessel.

Determination of Hydrogen-ion Concentration by Hildebrand's Method.[37] **Set up the apparatus as indicated in Fig. 9. The battery may be an ordinary dry cell. The voltmeter *V* should have a range of 1.2 volts and scale divisions to 0.01 volt. The portable galvanometer *G* should have a sensitivity of at least one megohm. A capillary electrometer may be used in place of the galvanometer. The rheostat *R* may be a tubular wire rheostat having at least 150 turns of wire. *K* is a contact key for opening and closing the circuit. *C* is**

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[36] Assemblies suitable for various purposes may be obtained from manufacturers and dealers. The equipment manufactured by Leeds and Northrup Co., Philadelphia, has been widely used.

[37] Hildebrand: *J. Am. Chem. Soc.*, 35, 847 (1913).

the calomel electrode.[38] *H* is a hydrogen electrode of the Hildebrand type especially suitable for solutions not containing protein. The Clark electrode is a type frequently employed in biochemical work and may be used with a shaking device to assist in obtaining equilibrium rapidly.

The essential part of the hydrogen electrode is the piece of platinum foil which is to dip into the unknown solution. This must be given a spongy coat of platinum black so that it will retain as much hydrogen as possible and must be saturated with hydrogen just before use.[39]

The unknown solution is placed in beaker *A*. The calomel electrode is clamped in place, as also is the prepared hydrogen electrode. A stream of hydrogen[40] is passed through the side arm of the hydrogen electrode so that it bubbles up through the solution at the rate of about two bubbles per second. Beaker *B* contains saturated KCl solution. The connecting tube contains saturated KCl made up in warm 3 per cent agar and allowed to solidify in the tube. This prevents syphoning between *B* and *A*.

Stir the solution well: an automatic stirring device is best for this purpose. Move the sliding contact on the rheostat until the voltage drawn from the dry cell is equal to that produced in unknown solution. This is indicated by the fact that no current passes through the galvanometer when the contact key *K* is pressed down, and there is therefore no deflection of the needle. Until a balance is reached the spring contact key should be closed only momentarily by a slight tap. At the balance point read the voltmeter.

CALCULATION OF pH. See the discussion preceding *Determination.* At 25° using saturated calomel electrode $\text{pH} = (E - 0.246)/0.059$. If for example the voltmeter reads 0.652 then $\text{pH} = (0.652 - 0.246)/0.059 = 7.0$ and the solution possesses a neutral reaction. Corrections for different electrodes and temperatures are given in the following table. This apparatus should have an accuracy of about 0.1 pH. Test the apparatus first, using buffer solutions of known pH.[41]

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[38] Calomel Electrode: Place about 3 ml. of carefully purified mercury in the bottom of the electrode vessel. Rub together mercury and mercurous chloride (calomel) with a little saturated KCl solution to form a paste. Place a layer of this paste about a half inch thick over the mercury and fill the vessel to the end of the side tube with saturated KCl solution. If it is desired to make a normal or tenth-normal calomel electrode corresponding solutions of KCl should be used instead of the saturated solution. It is convenient to make a permanent connection between the upper side arm and a reservoir bottle containing KCl solution. By running a little of the solution through the calomel cell before each series of determinations the side arm may be washed free from materials that may have diffused up into it. The stopcock of this electrode should be tight but should not be greased. A typical calomel electrode is shown in *C*, Fig. 9.

[39] Clean the platinum electrode thoroughly with chromic acid. Connect with the negative pole of a dry cell and connect another strip of platinum with the positive pole. Dip both in a dilute (2 per cent) solution of platinic chloride. Electrolyze for 10 to 15 minutes, frequently reversing the current. Dip into 10 per cent sulfuric acid, with the electrode as the cathode, and let the current run for a few minutes. The platinum is saturated with hydrogen. Wash with distilled water. Keep under distilled water when not in use. The platinum coat should last for several weeks but must be saturated with hydrogen just before use. Instead of a strip of platinum a wire about 1 mm. in diameter slightly flattened at the end may be used. Equilibrium with such an electrode may be brought about more rapidly. Shorter periods of electrodeposition produce electrodes which come to equilibrium more rapidly but which are not so permanent.

[40] Hydrogen may be purchased in cylinders, or may be prepared by electrolysis of an NaOH solution or from pure zinc. To purify pass through a wash bottle containing alkaline pyrogallol solution and two bottles containing water. Connect one of these bottles with a third tube dipping below the surface of mercury in another vessel. This acts as a safety. With gas tanks a gas regulator for adjusting flow is desirable.

[41] A suitable solution tenth-normal with respect to both acetic acid and sodium acetate may be used. At 25° with the saturated calomel electrode this gives 0.519 volt and with the 0.1 N electrode 0.6108 volt. Mix 50 ml. N NaOH and 100 ml. N acetic acid with water to make 500 ml.

STANDARD VALUES FOR CALOMEL ELECTRODES
(Referred to the Normal Hydrogen Electrode)

Temperature °C.	*E_0 for Different Concentrations of KCl*			$2.303 \frac{RT}{nF}$
	0.1 M	*1.0 M*	*Saturated (Approximate Potential)*	
18	0.3380	0.2864	0.2506	0.0577
20	0.3379	0.2860	0.2492	0.0581
25	0.3376	0.2848	0.2464	0.0591
30	0.3372	0.2836	0.2437	0.0601
40	0.3360	..	...	...

The hydrogen electrode under carefully defined conditions is the ultimate standard of reference for the determination of hydrogen-ion concentration in aqueous solution. The technical difficulties associated with its routine use, as well as its limitations under certain conditions, have led to a search for other electrodes suitable for the electrometric determination of pH. Among those which have found use may be mentioned the antimony electrode[42] the quinhydrone electrode, and the glass electrode. The glass electrode has superseded all others for most purposes.

The Glass Electrode. One type of glass electrode in common use consists of a bulb of special glass which is filled with some standard electrolyte, such as 0.1 N HCl, in contact with a suitable metallic electrode. When this bulb is immersed in an unknown solution a potential difference develops between the two solutions, the magnitude of which depends upon the hydrogen-ion concentration of the solution. This potential difference is measured as with the hydrogen electrode by combining the glass electrode with some standard half cell, such as the saturated calomel electrode, and measuring the voltage of the system. Because of the high resistance of the glass, however, the usual methods of determining the voltage, such as those used with the hydrogen electrode, cannot be used. Instead, a vacuum-tube amplifier is inserted in the potentiometer circuit. Various types of apparatus (*pH meters*) using this type of amplification and calibrated to read directly in pH units are available. Since they make use of various types of electrodes, amplification circuits, etc., the literature supplied with each instrument should be consulted for further details concerning the construction and operation of that instrument.

In general the determination of pH with a pH meter is quite simple. The solution is placed in a small container and the glass electrode and calomel electrode immersed in the solution. The potentiometer circuit is closed and adjusted until the null point is reached. The pH may then be read directly. Two general precautions are ordinarily necessary. The pH meter must be calibrated frequently by checking against a standard buffer of known pH, readjusting the scale setting if necessary. Failure to do this is the most frequent cause of error with a pH meter, since the calibration may change significantly from time to time, particularly if the glass electrode is new or has been allowed to become dry. It is best to keep both glass and calomel electrodes immersed in water when not in use. The temperature of the solution will also influence the determination of pH. In the Beckman pH meter illustrated (Fig. 10), for example, the temperature control must be set at the temperature of the unknown solution before the reading is made.

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[42] For review, see Perley: *Ind. Eng. Chem., Anal. Ed.*, **11**, 316 (1939).

The glass electrode is used for determinations of pH in all types of biological systems. Since (unlike the hydrogen electrode) it does not cause gas to bubble through the solution, it may be used on systems like blood which contain dissolved CO_2. The sample is not contaminated, so that the method is especially applicable where the amount of material is limited. Electrodes are available which require only a few tenths, or even hundredths, of a milliliter of solution for each determination. Determinations with the glass electrode are rapid, and with most instruments the accuracy is within 0.05 pH. It may be used on unbuffered, colored, or turbid solutions and solutions containing sediments. With the ordinary glass electrode, determinations are accurate up to a pH of 10 or even higher, provided the solutions do not contain appreciable quantities of sodium, lithium, or potassium ions. Sodium ions, especially, exert a considerable effect on determinations in solutions with a pH of 9 or above, when they are present in a concentration of normal or greater. Corrections may be applied in such cases (Dole). Special electrodes have been

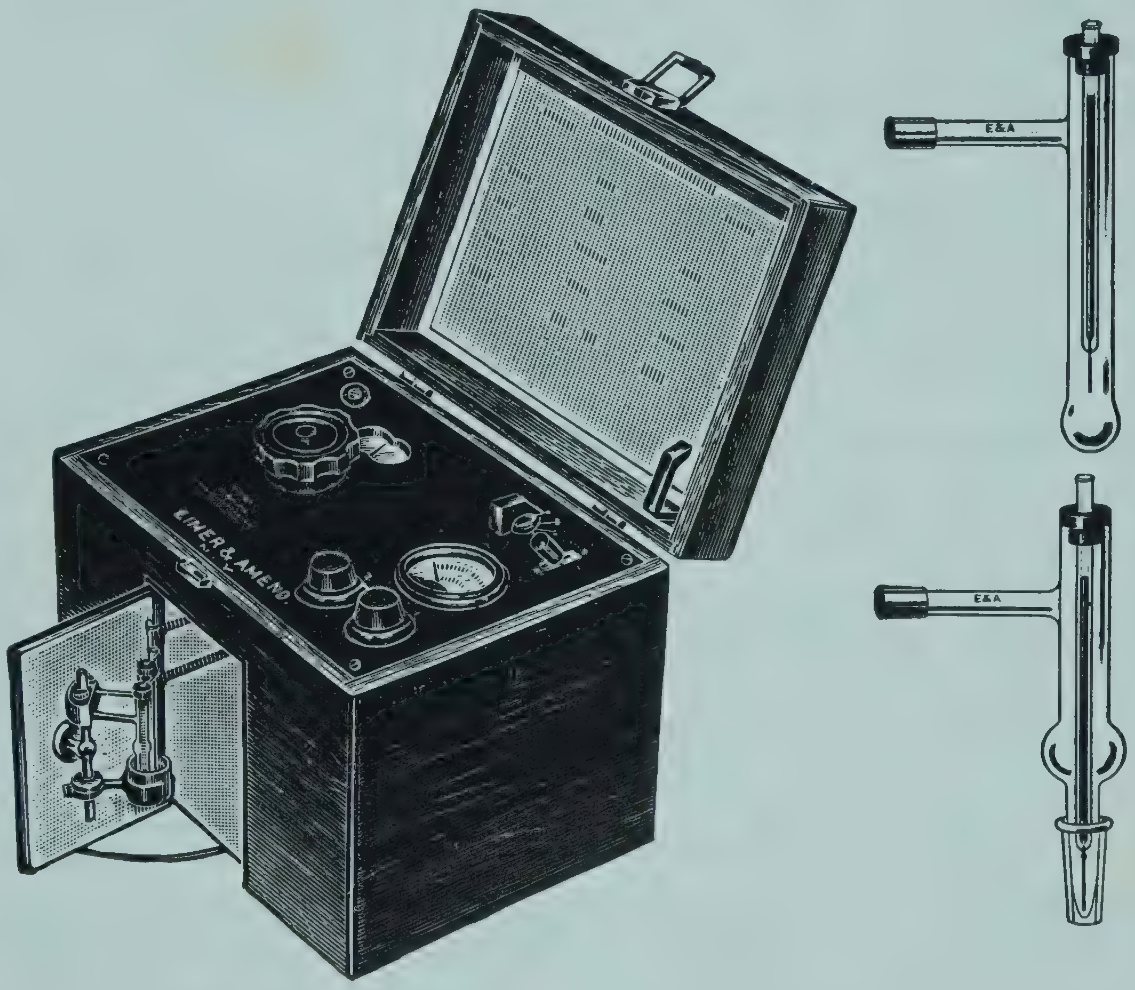

FIG. 10. GLASS-ELECTRODE APPARATUS (BECKMAN).
Glass electrode shown at right (*above*) and calomel electrode (*below*).

developed for use in strongly alkaline solutions, for solids such as cheese, and for many other purposes.

Electrometric Titration of Acids and Bases. In many cases the use of indicators in titration is unsatisfactory because of color or turbidity of solutions to be examined or the presence of buffer substances which make the end point uncertain. The hydrogen or glass electrodes may be used in such cases in titrating solutions to a definite acidity or alkalinity. This method also makes it possible to follow the changes in acidity during the course of a titration and gives information as to the character of the acids or bases in solution. Some applications of this method are illustrated in Fig. 11.

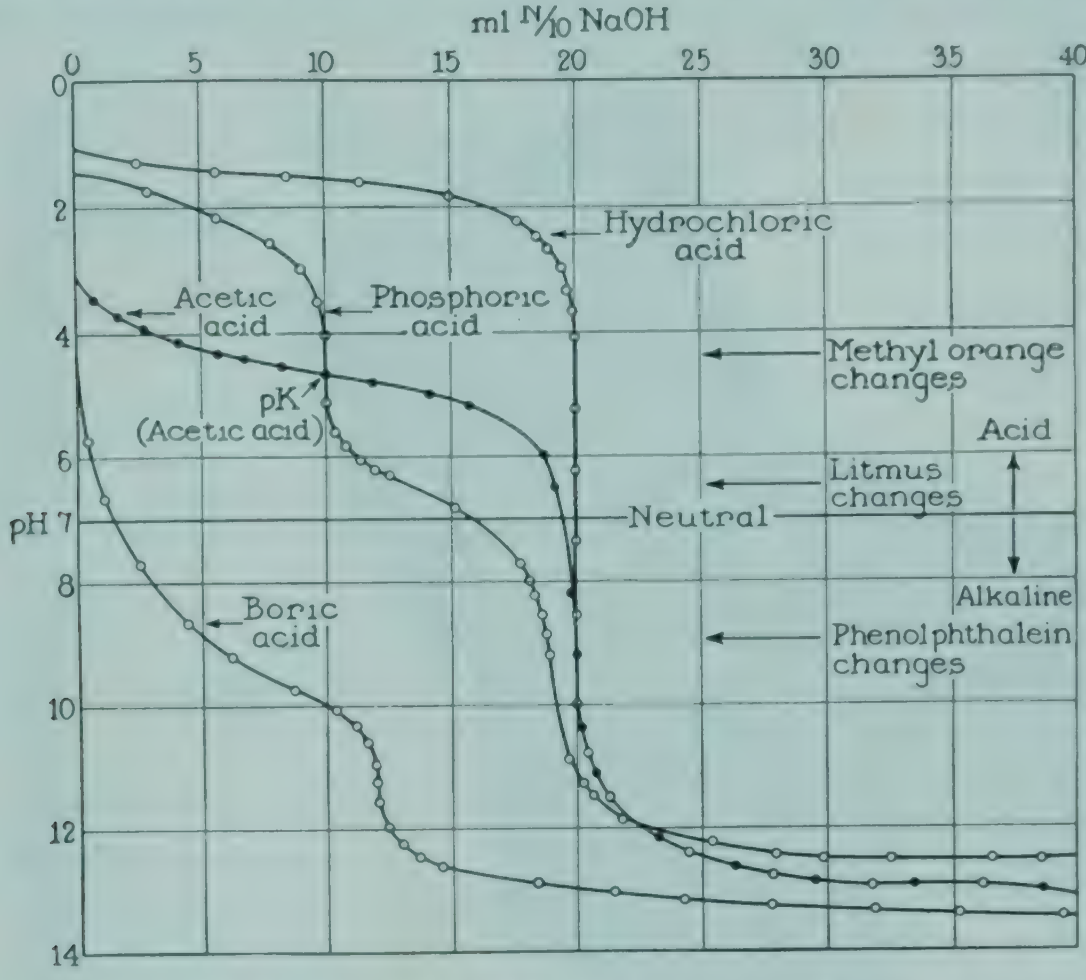

Fig. 11. Titration Curves of Various Acids, Obtained by Electrometric Measurement.

Twenty ml. of 0.1 N hydrochloric and acetic acids used. *Phosphoric acid* about ⅔ molar. *Boric acid*, saturated solution.

Procedure. The apparatus already described and illustrated in Fig. 9 may be used. The unknown solution is placed in beaker A, into which are introduced both the hydrogen and calomel electrodes. Beaker B and the connecting tube are omitted. Hydrogen is bubbled through the solution and acid or alkali added from a buret with stirring. At intervals read the voltmeter. Plot a curve of voltage or pH against milliliters of acid or alkali added. Neutralization is indicated by a rapid change of voltage or pH. Di- or tribasic acids or bases may show two or more such changes. See the curve for titration of phosphoric acid (Fig. 11).

The glass-electrode pH meter is particularly suitable for this purpose. Special electrodes with long leads are available, for direct titration in an ordinary beaker.

As will be seen from Fig. 11, in the titration of a strong acid such as hydrochloric acid with a strong base such as sodium hydroxide, the change in $[H^+]$ at the end point is very sharp because, as long as even 0.1 ml. of the acid remains unneutralized, the pH is still quite low and 0.1 ml. of 0.1 N NaOH in excess gives a very large increase of pH. Evidently either methyl orange, litmus, or phenolphthalein may be used in the titration. With acetic acid however the beginning $[H^+]$ is lower because this acid is weakly dissociated. The change in pH is at first fairly rapid because the sodium acetate formed yields acetate ions which repress the ionization of the acid. As the titration is continued the ionization of the acid is almost completely repressed by the larger amounts of acetate formed. When an equivalent amount of alkali has been added, the reaction is alkaline owing to slight hydrolysis of the sodium acetate to form acetic acid (nearly all undissociated) and highly dissociated NaOH. The actual end point of the titration is therefore on the alkaline side and phenolphthalein is the proper indicator.

Fig. 11 shows also how it is possible to titrate the first hydrogen of phosphoric acid using methyl orange as an indicator and the second using phenolphthalein; the third cannot be titrated because the change is too gradual. Note also that boric acid cannot be titrated with phenolphthalein as an indicator.

Electrometric Oxidation and Reduction Titrations. When an ion is oxidized it loses electrons or negative charges; when it is reduced it gains electrons. Thus when Fe^{++} is oxidized to Fe^{+++} there is a loss of one electron. The process is therefore similar to that resulting in the loss of an electron when hydrogen passes into solution as H^+. If a platinum wire is placed in a solution containing ferrous and ferric ions, the ferrous ions tend to give up electrons to the platinum and the ferric ions tend to take them away. A difference of potential will arise depending upon the concentrations of the two ions. If an oxidizing agent is now added there will be a change of potential, slow at first and then very rapid, as the concentration of ferrous ions becomes very small compared to that of the ferric ions. This rapid change of potential marks the end point of the titration.

Procedure. **The apparatus used is the same as for the electrometric titration of acids and bases. The platinum electrode should however be small and should not be platinized but be bright, and kept in 1:1 HCl when not in use. The calomel electrode should be connected to the negative side of the main circuit instead of the positive. Instead of a beaker a flask may be used and a current of CO_2 passed over the surface of the solution (not through it) to prevent oxidation by the air.[43] Run in an oxidizing or reducing agent from a buret. Readings of the voltmeter are taken at intervals and at the end point a drop or two of solution produces a marked change of potential.**

An electronic pH meter with the glass electrode replaced by a platinum electrode may also be used. See the manufacturer's directions for the proper connection and use.

The Polarograph. Instead of adding a reactant to the solution and using the change in potential at an electrode for quantitative purposes as

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[43] If desired the voltmeter may be eliminated. In this case adjust the rheostat so that there is no galvanometer deflection at the beginning of the titration. The end point is indicated by the galvanometer needle being thrown off the scale.

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described above, it is possible to apply an external voltage across the electrodes and electrolyze the ions by forcing them to react at the electrodes. At the cathode for example the univalent metallic ion M^+ will accept an electron ϵ and be reduced to the metallic state:

$$M^+ + \epsilon = M$$

Under the proper conditions the voltage required to bring about such a reaction is characteristic of the chemical nature of the ion, and the current resulting from electron transfer may be used as a measure of the concentration of the ion.

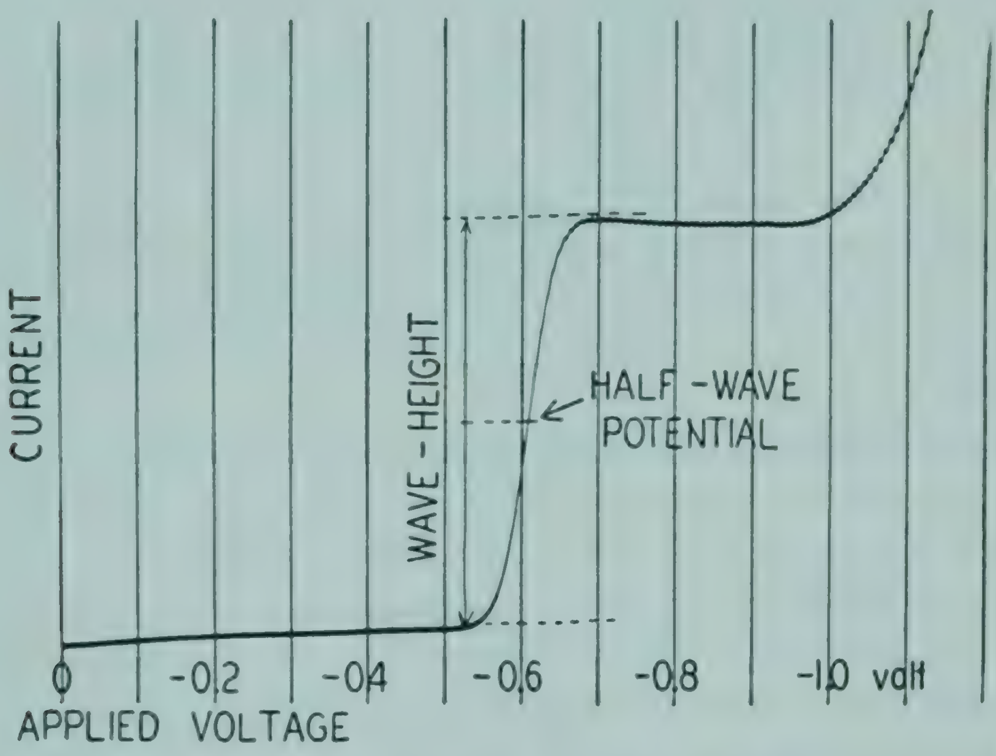

FIG. 12. TYPICAL POLAROGRAM.

Air-free solution of 0.001 M $CdSO_4$ in 0.1 M $CoCl_2$ buffered at pH 5.

From Müller: *J. Chem. Education*, 18, 71 (1941).

The instrument employed to apply a known and variable voltage across the electrodes, and to measure the resulting current, is called the polarograph (Heyrovsky). A simple form of polarograph which can be assembled from equipment available in most laboratories has been described by Müller[44]. The graphical recording of the relation between applied voltage and current is called a polarogram (Fig. 12). Most commercial polarographs[45] provide for the continuous and automatic recording of the polarogram, using automatic recorders or photography.

In its simplest form, the current-voltage curve for a particular ion is approximately symmetrical around the half-wave potential, which is determined by the kind of ion undergoing reduction. Under the proper conditions the wave height becomes a measure of the concentration of the ion. The concentration of material required for polarographic analysis is quite low, ranging from 10^{-3} M to 10^{-5} M under ordinary circumstances in a volume of 1 ml. or more. It is worthy of note that solutions may be regarded as essentially unchanged in composition after polarography, since the actual amount of electrolysis which occurs is ordinarily almost undetectable in terms of concentration.

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[44] Müller: *J. Chem. Education*, 18, 112 (1941).

[45] Manufacturers of polarographs include E. H. Sargent Co. of Chicago, Leeds and Northrup of Philadelphia, Fisher Scientific Co. of New York, American Instrument Co. of Silver Springs, Md., and others.

In addition to its obvious application to the field of inorganic analysis, the polarograph is finding increasing application in organic and physiological chemistry. It has been applied for example to the determination of certain hormones and vitamins, to a differentiation between normal and pathological blood serum, and to the measurement of oxygen consumption by plant and animal cells.

Electrical Conductance of Solutions. The power of a solution to conduct an electrical current depends on the number of ions in the solution and the mobility of those ions. The conductance of a solution is determined by placing the solution between platinum electrodes of known area in a conductivity cell and measuring the electrical resistance of this cell in a Wheatstone-bridge arrangement; details of this procedure can be found in any standard textbook on physicochemical methods. The *conductance* is the reciprocal of the resistance, and is expressed in reciprocal ohms, or *mhos*. Special conductivity cells have been devised for biological measurements, including one type which is built into a stomach tube for studying the conductance of the gastric juice.

The conductance of most biological fluids is largely dependent upon their content of sodium, potassium, chloride, and bicarbonate ions. In the gastric juice, which contains hydrochloric acid, the hydrogen ion is important in this connection. Differences between various body fluids in electrical conductance are due chiefly to differences in the electrical mobility of the ions present, since there is relatively little variation among body fluids in total electrolyte content. Thus the specific conductance of bile at 18° is 130×10^{-4} reciprocal ohms, that of blood serum about 110×10^{-4}, and that of saliva about 50×10^{-4}, whereas the conductance of pure gastric juice is over 400×10^{-4} reciprocal ohms. In total chlorine, gastric juice is not notably higher than blood serum but it conducts a current much more readily because of the great mobility of the hydrogen ion. The ionic speed (at 18° and a potential of one volt per cm.) of the sodium ion is 43.5×10^{-5} cm. per second, of the potassium ion 64.6×10^{-5}, of the chloride ion 65.5×10^{-5}, whereas that of the hydrogen ion is 318×10^{-5} cm. per second. Conductivity determinations have been used for a variety of biological purposes, as for the measurement of the volume of red cells in blood (the plasma conducts, but the red cells do not) and for other studies on blood and urine. Studies on suspensions of other types of cells also throw light on the conductivity of protoplasm and the permeability of the cells for ions.

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2

Carbohydrates

Definition. The name carbohydrates is given to a class of substances which are especially prominent constituents of plants and are found also in the animal body, either free or as components of certain proteins, lipides, and other compounds. They are called carbohydrates because they contain the elements C, H, and O, the H and O being present in the proportion to form water. The term is not strictly appropriate inasmuch as there are compounds (such as acetic acid, lactic acid, and inositol) which have H and O present in the proportion to form water, but which are not carbohydrates, and there are also true carbohydrates which do not have H and O present in this proportion—e.g., rhamnose, $C_6H_{12}O_5$. Chemically considered the carbohydrates are aldehyde or ketone derivatives of polyhydric alcohols, or condensation products of such substances. The aldehyde derivatives are spoken of as aldoses, and the ketone derivatives are spoken of as ketoses. It is worthy of note that among pure organic compounds, the one prepared in the largest quantity in the United States is sucrose, a carbohydrate.

Classification. The carbohydrates are usually classified, according to the number of simple carbohydrate groups which they contain, as mono-, di-, tri-, and polysaccharides. The monosaccharides, which contain only a single such group and cannot therefore be hydrolyzed into simpler substances, are further characterized according to the length of the carbon chain as trioses ($C_3H_6O_3$), tetroses ($C_4H_8C_4$), pentoses ($C_5H_{10}O_5$), hexoses ($C_6H_{12}O_6$), etc. The disaccharides give two molecules of simple sugars on hydrolysis and the more common ones have the general formula $C_{12}H_{22}O_{11}$. The polysaccharides give many molecules of simple sugars on hydrolysis and the formula for the more important members of this group is $(C_6H_{10}O_5)_x$ where x represents the number of simple sugar groups present. In a general way the solubility of the carbohydrates varies with the complexity, the more complex being the less soluble. This means simply that, as a class, the monosaccharides (hexoses) are the most soluble and the polysaccharides (starches and cellulose) are the least soluble.

The more common carbohydrates may be classified as follows:

I. Monosaccharides.
    1. Hexoses ($C_6H_{12}O_6$). Glucose, fructose, galactose, mannose.
    2. Pentoses ($C_5H_{10}O_5$). Arabinose, xylose, ribose, rhamnose (methyl-pentose) $C_6H_{12}O_6$, deoxyribose ($C_5H_{10}O_4$).

II. Disaccharides ($C_{12}H_{22}O_{11}$).
    Maltose, lactose, sucrose, gentiobiose, isomaltose, cellobiose.

III. Trisaccharides ($C_{18}H_{32}O_{16}$).
    Raffinose.
IV. Polysaccharides ($(C_6H_{10}O_5)_x$).
    1. Starch Group. Starch, dextrin, glycogen, inulin.
    2. Cellulose Group.
        (a) Cellulose.
        (b) Hemicelluloses.
            (1) Pentosans. Gum arabic.
            (2) Hexosans. Galactans; agar-agar; dextrans; levans.
            (3) Hexo-pentosans. Pectin.

Photosynthesis. It is interesting to note that the bulk of these highly complex carbohydrates found in nature, whose structures in many instances are as yet unknown, are synthesized by plants from such simple precursors as water and carbon dioxide. Although the mechanism of such synthesis is still obscure, it has long been recognized that the synthetic process is related to the presence of sunlight and chlorophyll or other such coloring matter in the plant.

It is generally agreed that photosynthesis occurs in two distinct phases, one involving reactions which proceed in the absence of light (*dark reaction* or *Blackman reaction*), and the other involving the presence of light and a photosensitive catalyst such as chlorophyll or other pigment. Opinions vary as to the relative contributions of these two phases to the total process; earlier views postulated that carbon dioxide utilization took place in the dark, but oxygen evolution was associated with the presence of light and chlorophyll. More recent views incline to the belief that both carbon dioxide assimilation and oxygen production are dark reactions in the sense that they do not fundamentally require light, the function of the light being to initiate and maintain the sequence of dark reactions by the photochemical decomposition of a suitable substance, possibly water itself (see below), in the presence of the photocatalyst chlorophyll or similar pigment.

The simplest possible formulation of the over-all reaction for the production of a carbohydrate such as glucose ($C_6H_{12}O_6$) from carbon dioxide and water by photosynthesis is the following:

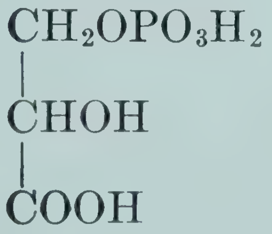
$$CO_2 + 2H_2O \rightarrow [CH_2O] + H_2O + O_2$$
$$6[CH_2O] \rightarrow C_6H_{12}O_6$$

The postulated intermediate compound $[CH_2O]$ has the elementary composition of formaldehyde, and in fact it was suggested many years ago by Baeyer that formaldehyde was indeed the intermediate in the photosynthesis of carbohydrate. Although the reaction as shown does serve to explain the observed 1:1 ratio between carbon dioxide utilization and oxygen evolution during photosynthesis, there is little positive evidence that formaldehyde or its equivalent is concerned in the processes of photosynthesis, despite the large amount of work which has been done in this connection.

Water is shown as both reactant and product, since evidence obtained by the use of the oxygen isotope O^{18} (*heavy oxygen;* see p. 983) as a tracer indicates that the oxygen evolved during photosynthesis is derived from the water entering the reaction and not from the carbon dioxide utilized. Furthermore, the oxygen of the water that is formed is derived from the carbon dioxide. Hence the reaction is essentially a reduction, water supplying both the hydrogen required for the reduction and the oxygen which is evolved. According to this view, the role of water in the photochemical process may be visualized as follows:

$$\begin{tikzcd} & \text{Hydrogen for reduction} \\ H_2O + \text{Light energy} \arrow[ur] \arrow[dr] & \\ & \text{Oxygen} \end{tikzcd}$$

The availability of carbon dioxide labeled with radioactive carbon has stimulated research into the mechanisms of carbon dioxide assimilation during photosynthesis. The results of Calvin and his associates are of particular interest in this connection. Using various green plants (grains, algae), these investigators were able to show that, following a brief period of photosynthesis in the presence of radioactive carbon dioxide, a number of compounds containing radioactive carbon could be isolated from the plant extracts and identified. *Paper chromatography* (see p. 16) proved to be of particular value in these experiments. As the period of photosynthesis was made progressively shorter, until it was reduced to a matter of only a few seconds' exposure to light, the number of different labeled compounds isolated was reduced until only one substance stood out as a major bearer of the assimilated radiocarbon; this substance is phosphoglyceric acid:



$$\begin{array}{l} CH_2OPO_3H_2 \\ | \\ CHOH \\ | \\ COOH \end{array}$$

Phosphoglyceric acid

The radiocarbon is found more abundantly in the carboxyl group of the phosphoglyceric acid than in the other two carbon atoms; this leads to the belief that the first step in CO_2-uptake during photosynthesis is the condensation of CO_2 with some 2-carbon precursor to form phosphoglyceric acid. The nature of this precursor is not known; the finding of the isotope label in the other two carbon atoms of phosphoglyceric acid, but to a lesser extent, indicates that the precursor is produced by some cyclic process yet to be worked out in which phosphoglyceric acid itself plays a part; a tentative cycle involving 5-carbon and 7-carbon sugars as intermediates has been proposed, but the details remain obscure.

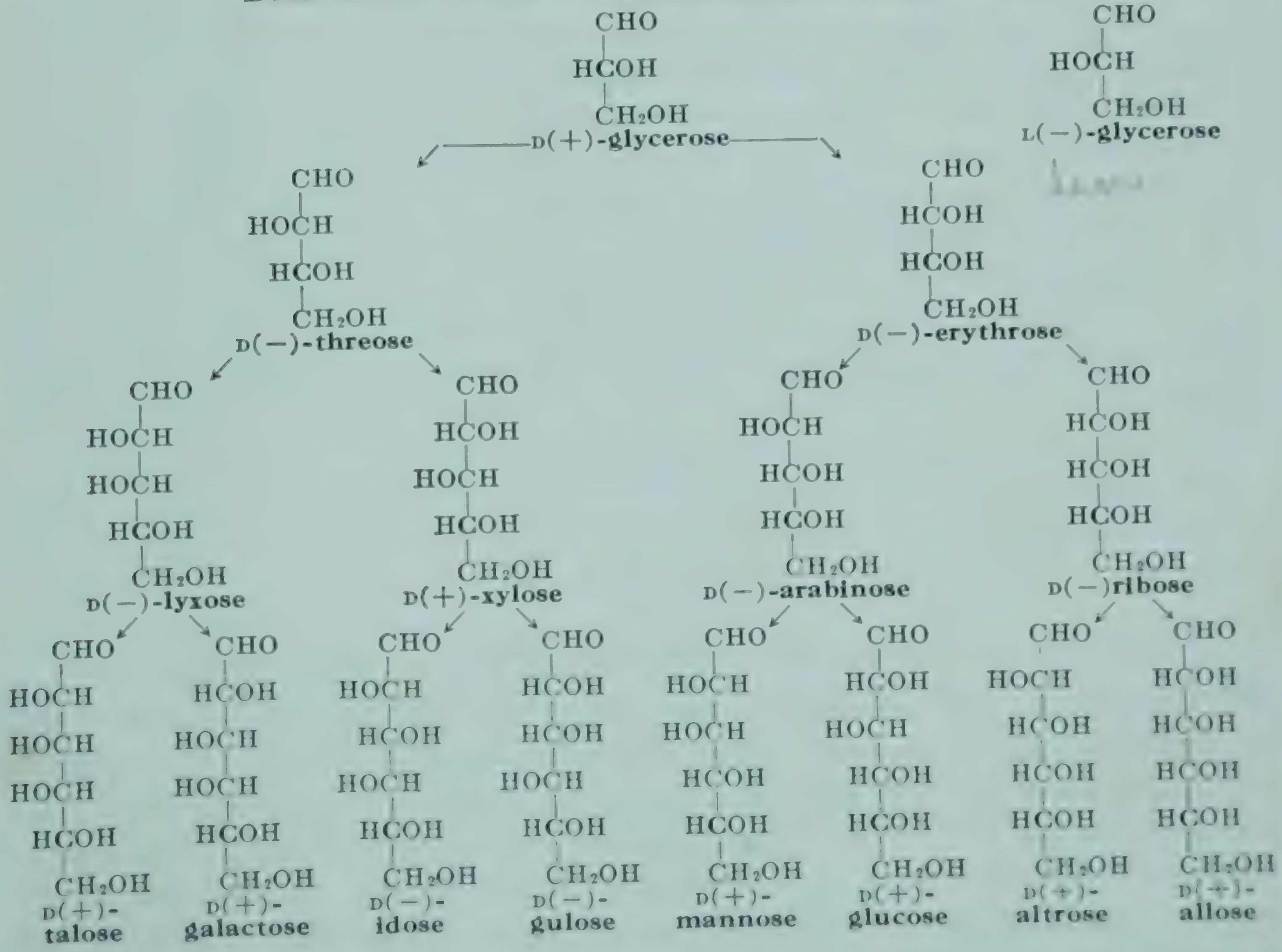
Phosphoglyceric acid is a well-recognized intermediate in the breakdown of carbohydrate by animal tissues (see p. 275). Indeed, the student of animal biochemistry will note a striking resemblance between the reactions of plant photosynthesis thus far described and those already known for carbohydrate metabolism in animal tissues. Furthermore, CO_2-

assimilation also occurs in animal tissues to a limited but definite extent, as discussed in Chapter 33, and there appears to be much in common between the processes as they occur in plants and animals. The action of light in photosynthesis remains obscure; according to one view, the light energy acts by opening the S—S bond in a compound such as 6,8-thioctic acid (α-lipoic acid, see Chapter 35). This would then be the reduction reaction mentioned above; how this fits in with the water reaction remains to be established.

It should not be inferred that photosynthesis is of importance solely in connection with carbohydrates. Plants also produce nitrogenous and non-nitrogenous substances such as amino acids, proteins, and fats, which are of fundamental significance to animal nutrition, and there is no reason to believe that the energy of photosynthesis is not used directly or indirectly for the formation of these compounds also.

Spatial Configuration of the Sugars. The triose glycerose (also called glyceraldehyde) has one asymmetric carbon atom and therefore exists in two optically active forms, one dextrorotatory and the other levorotatory. The spatial arrangement of the groups around the asymmetric carbon atom of the dextrorotatory form of glycerose is arbitrarily called the D configuration,[1] while that for the levorotatory form is called the L configuration. The connotation of D and L refers to spatial configuration *only* and is *not* an indication of the direction of rotation of polarized

DERIVATION OF THE D-ALDOSES FROM D-GLYCEROSE



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[1] The carbohydrate nomenclature used in this chapter follows the recommendations of the Committee on Carbohydrate Nomenclature of the American Chemical Society.

light by the compound. If it is desired to indicate the direction of rotation for the compound, the symbols (+) meaning dextrorotatory and (−) meaning levorotatory, may be used. Thus a dextrorotatory compound with the D configuration may be indicated as D(+), while a levorotatory compound with the same configuration has the symbol D(−).

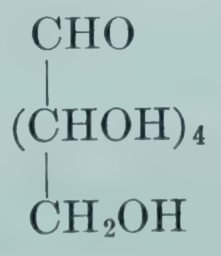
As the number of asymmetric carbon atoms in the sugar molecule increases on going from the trioses to the higher monosaccharides, the number of stereoisomers increases in accordance with the van't Hoff formula 2^n, where n represents the number of asymmetric carbon atoms. All of these stereoisomers which have a configuration identical with that of D-glycerose around a suitably selected *reference asymmetric carbon atom* are called D sugars, regardless of their direction of rotation. The reference carbon atom is, for sugars containing more than one asymmetric carbon, the asymmetric carbon atom farthest removed from the active (i.e., aldehyde or ketone) end of the molecule. These relationships for the D aldoses are illustrated by the preceding chart, in which the reference asymmetric carbon atom is indicated by heavy black type. In this connection note that if the structure is written with the active group at the top it is conventional to write the OH group on the right-hand side of the reference asymmetric carbon atom for the D configuration and on the left-hand side for the L configuration. Naturally occurring sugars are mainly of the D configuration; L sugars have, however, been isolated from certain plant and animal sources.

MONOSACCHARIDES

HEXOSES, $C_6H_{12}O_6$

The hexoses are monosaccharides containing a chain of six carbon atoms in the molecule. They may be either aldoses or ketoses. They are among the most important of the simple sugars and occur widely distributed in nature, either in the free state or in combination with other molecules, from which they may usually be separated by hydrolysis. The most important hexoses biologically are glucose, fructose, galactose, and mannose. Of these, fructose is a ketohexose; the others are aldohexoses. The various aldohexoses differ structurally from one another solely in the spatial arrangement of the H and OH groups around certain of the carbon atoms in the molecule (see p. 58). This difference may result in markedly different physiological properties. As a class the hexoses are extremely soluble, are optically active, and possess certain characteristic general and specific chemical properties which are used in their identification and determination.

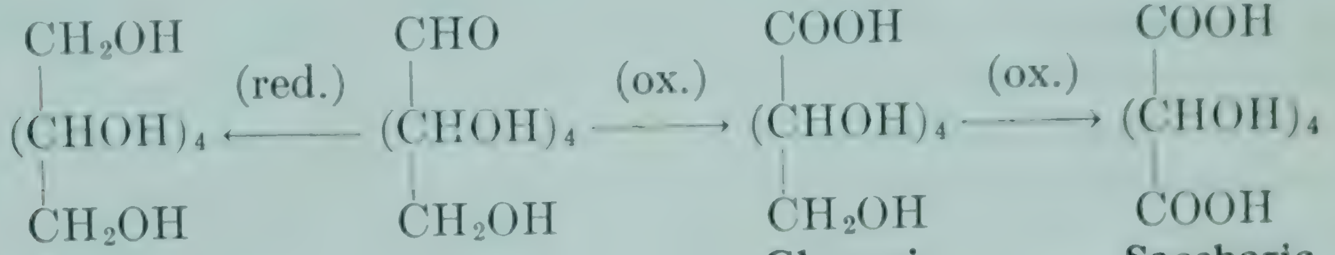
GLUCOSE



$$\begin{array}{c} \mathrm{CHO} \\ | \\ (\mathrm{CHOH})_4 \\ | \\ \mathrm{CH_2OH} \end{array}$$

Glucose, also called dextrose or grape sugar, is found widely distributed in nature either in the free state or combined with other compounds. Together with fructose, it occurs in the juice of many fruits, and is obtained commercially by the hydrolysis of starch. It is the sugar found in the blood, where its concentration normally is about 0.1 per cent. Ordinarily it is not present in the urine except possibly in traces, but appreciable amounts are found under certain conditions, such as in diabetes mellitus.

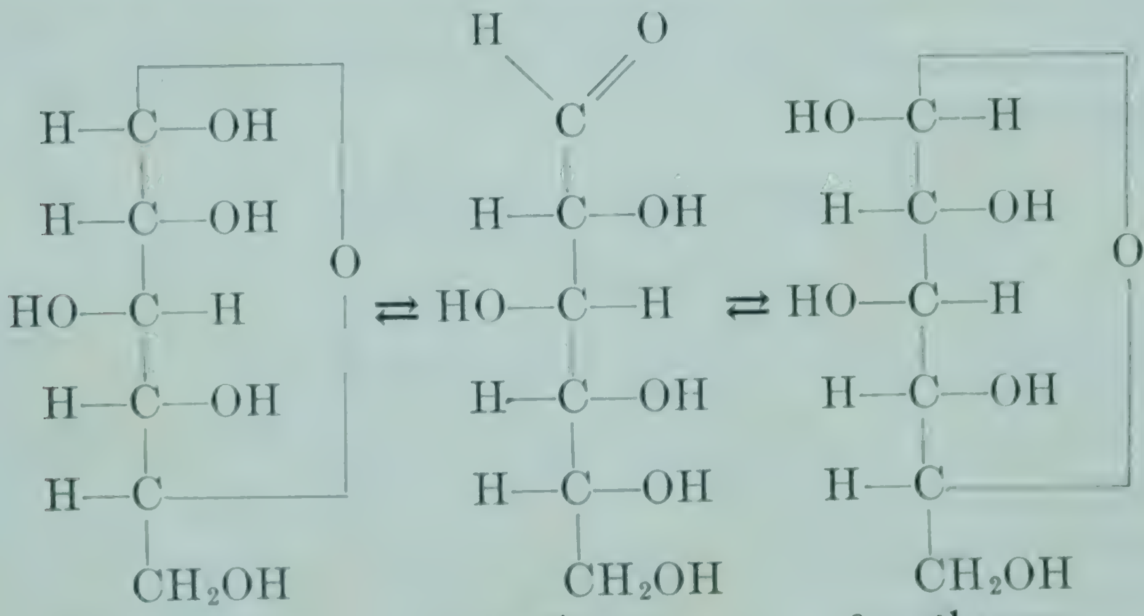
It is dextrorotatory in solution, having a specific rotation of +52.5°. As an aldohexose it may be oxidized to the corresponding sugar acids or reduced to an alcohol.



$$\begin{array}{c}CH_2OH\\|\\(CHOH)_4\\|\\CH_2OH\end{array}\xleftarrow{\text{(red.)}}\begin{array}{c}CHO\\|\\(CHOH)_4\\|\\CH_2OH\end{array}\xrightarrow{\text{(ox.)}}\begin{array}{c}COOH\\|\\(CHOH)_4\\|\\CH_2OH\end{array}\xrightarrow{\text{(ox.)}}\begin{array}{c}COOH\\|\\(CHOH)_4\\|\\COOH\end{array}$$

Sorbitol **Glucose** **Gluconic acid** **Saccharic acid**

Another oxidation product, glucuronic acid, $CHO \cdot (CHOH)_4 \cdot COOH$ is of considerable physiological importance, being found in the urine in combination with certain excretory products (see Chapters 20 and 29).

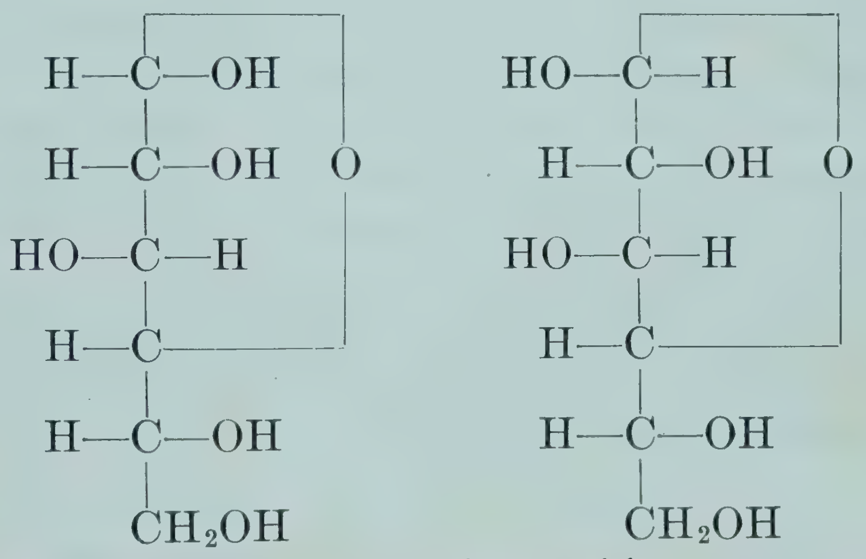


α-D-glucose **D-glucose (free aldehyde form)** **β-D-glucose**

The behavior of glucose solutions indicates that only a small fraction of the substance is present in the free aldehyde form, the remainder existing chiefly in a less active modification in which the carbon atom of the aldehyde group is linked to the fifth carbon atom by means of an atom of oxygen. (See formulas.) This type of linkage is known as the amylene oxide ring and since, in its formation, the first carbon atom has become asymmetric, we find that two modifications of this form exist: the first, known as α-D-glucose, has a specific rotation of +113.4° and the second, known as β-D-glucose, has a specific rotation of +19°. In solution these forms are in equilibrium with each other and probably also with a small amount of the more active form having the free aldehyde group, the specific rotation of the equilibrium mixture, which is the rotation ordinarily measured, being +52.5°.

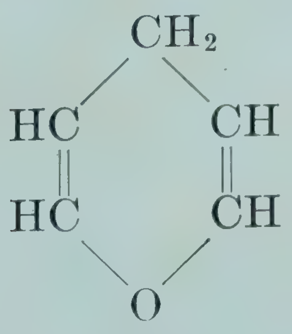
In freshly prepared solutions of the usual crystalline glucose the α form exists alone, the solution having a specific rotation of +113.4°. On standing, however, some of the molecules are slowly transformed into the β form and an equilibrium is finally established between the two forms. A trace of alkali hastens the formation of this equilibrium. The specific rotation of the solution falls as the amount of the β form increases until equilibrium, with its characteristic specific rotation of +52.5°, is reached. This phenomenon is known as *mutarotation*. Mutarotation is observed not only with glucose but with all sugars which have a free or potentially free aldehyde or ketone group in the molecule.

In addition to the amylene oxide configurations, glucose may exist in forms where the terminal carbon atom is linked through an oxygen atom to other carbon atoms than the fifth, thus forming ethylene, propylene, and butylene oxide rings each of which may exist in the α and β forms. Of these various forms the butylene oxide form predominates, but there is evidence that all of the forms mentioned may exist in glucose solutions. These modifications are more reactive than the ordinary amylene oxide form and are presumably responsible for the concept of an "active" form of the glucose molecule, the so-called γ-glucose.

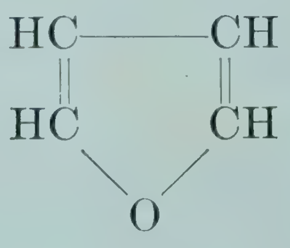


α and β forms, respectively, of butylene oxide structure of D-glucose

Haworth has pointed out that the amylene oxide and butylene oxide rings are structurally related to pyran and furan, respectively. Normal

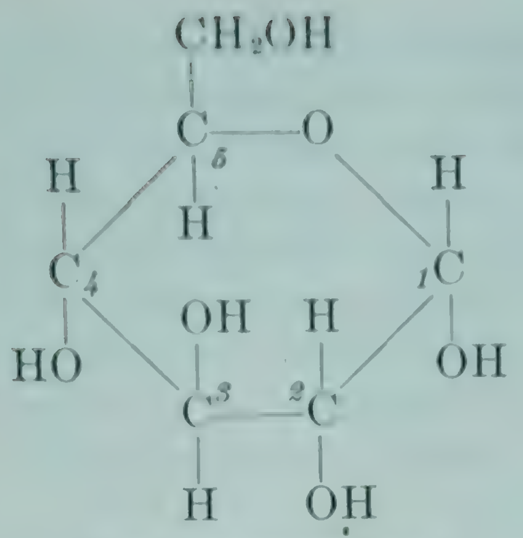


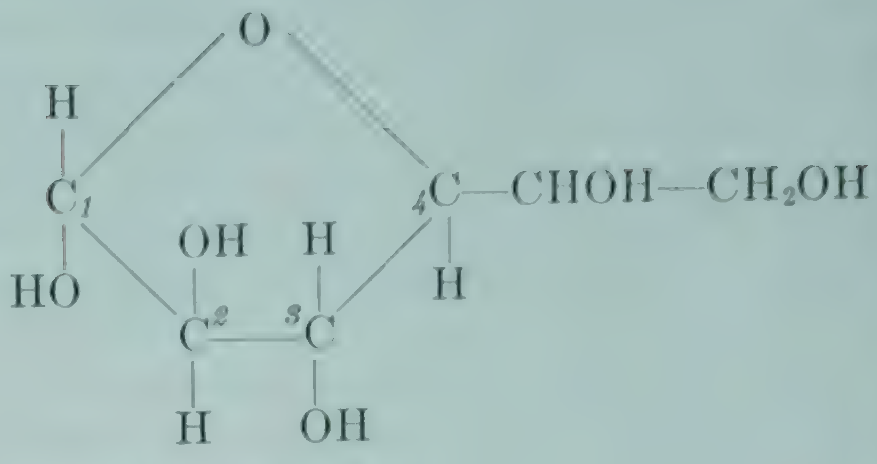
Pyran



Furan

glucose, using the nomenclature proposed by Haworth and now widely used, thus becomes *glucopyranose* while the butylene oxide form is called *glucofuranose.*


α-D-glucopyranose


β-glucofuranose

X-ray examinations indicate that the five carbon atoms of the pyranose ring lie in the same plane while the oxygen atom lies in a different plane. The H and OH groups are placed above and below this plane of carbon atoms in accordance with the chemical evidence concerning the positions of these groups in the sugar molecule.

In common with other reducing sugars, glucose is quite labile to the action of alkali. A trace of alkali accelerates the attainment of equilibrium between the α and β forms. Further contact with alkali results in the appearance of fructose and mannose in the solution in equilibrium with glucose, probably through the intermediate formation of a common enol tautomer involving the first two carbon atoms. Strong alkali (with heating) decomposes the molecule completely, producing a number of smaller fragments and condensation products which usually give a brown color to the solution (Moore's test, now obsolete).

Glucose is one of the sweetest of the common sugars, being excelled only by fructose and sucrose. The following table gives the relative sweetness of some of the sugars considering sucrose as 100:

Sugar	*Relative Sweetness (Sucrose = 100)*
Lactose	16.0
Raffinose	22.6
Galactose	32.1
Rhamnose	32.5
Maltose	32.5
Xylose	40.0
Glucose	74.3
Sucrose	100.0
Invert sugar	130.0
Fructose	173.3

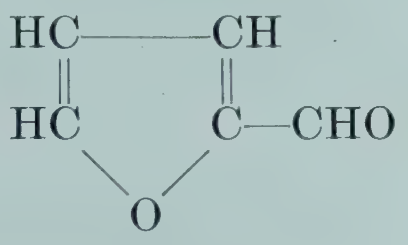
EXPERIMENTS ON GLUCOSE

The following tests are made on glucose (1 per cent solution) as a typical carbohydrate, and are *not specific for this sugar*. A specific test for glucose is the formation of glucosazone by the Phenylhydrazine Reaction (3, below) without the preliminary formation of an insoluble hydrazone (mannose, p. 75) and in the absence of a positive Resorcinol-Hydrochloric Acid Reaction (fructose, p. 73).

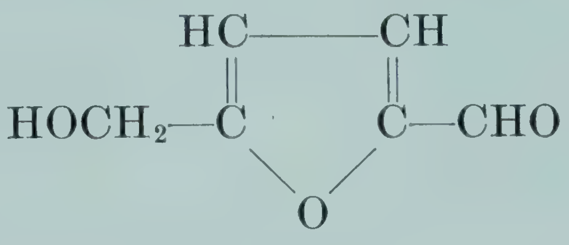
1. *Solubility.* Test the solubility of glucose in water and in alcohol. If in doubt about the solubility of a compound, filter from excess solid and test the filtrate for the substance in question; or if the solvent is nonaqueous, allow it to evaporate and examine it for a residue.

2. *α-Naphthol Reaction* (*Molisch*). To 5 ml. of sugar solution in a test tube, add 2 drops of Molisch's reagent (a 5 per cent solution of α-naphthol in alcohol). Mix thoroughly. Incline the tube and allow about 3 ml. of concentrated sulfuric acid to flow down the side of the tube, thus forming a layer of acid beneath the sugar. A reddish-violet zone appears at the junction between the two liquids. Repeat the test, using 5 ml. of 0.1 per cent furfural solution instead of the sugar. Instead of α-naphthol, 3 to 4 drops of a 5 per cent alcoholic solution of thymol may be used.[2]

The reaction is due to the formation of furfural and furfural derivatives, such as hydroxymethylfurfural, by the acid acting on the sugar.



Furfural



Hydroxymethylfurfural

The test is thus given by furfural and all furfural-yielding substances and is not a specific test for carbohydrates. Concentrated solutions of organic compounds may give a red instead of a violet color due to the charring action of the sulfuric acid. In case of doubt the reaction should be repeated on a more dilute solution of the material to be tested.

3. *Phenylhydrazine Reaction.* To a small amount of phenylhydrazine mixture (about 0.5 inch in a small test tube),[3] add 5 ml. of the sugar solution, shake well, and heat on a boiling water bath for one-half to three-quarters of an hour. Allow the tube to cool slowly (not under the tap) and examine the crystals microscopically (see Plate II). Better crystals may be obtained if the tubes are allowed to cool in the water bath.

If the solution has become too concentrated in the boiling process it will be light red in color and no crystals will separate until it is diluted with water.

Yellow crystalline compounds called osazones are formed from certain sugars under these conditions, each individual sugar in general giving rise to an osazone of a definite crystalline form which is typical for that sugar. It is important to remember in this connection that, of the simple sugars of interest in physiological chemistry, glucose, fructose, and mannose yield the same osazone because of similarities in their molecular structures. Of the various osazones, it is generally possible to recognize glucosazone by its crystalline form. Maltosazone may also be recognized if it happens to crystallize in its most characteristic form. Otherwise it may not be possible to distinguish it from lactosazone. The melting points of the recrystallized osazones may be used as further means of identification, but since they extend over a range of several degrees and are not far apart for the different sugars, the determination of melting points is of doubtful value.[4] The reaction with glucose is sufficiently delicate to produce under favorable conditions a visible precipitate with solutions containing as little as 0.01 per cent glucose.

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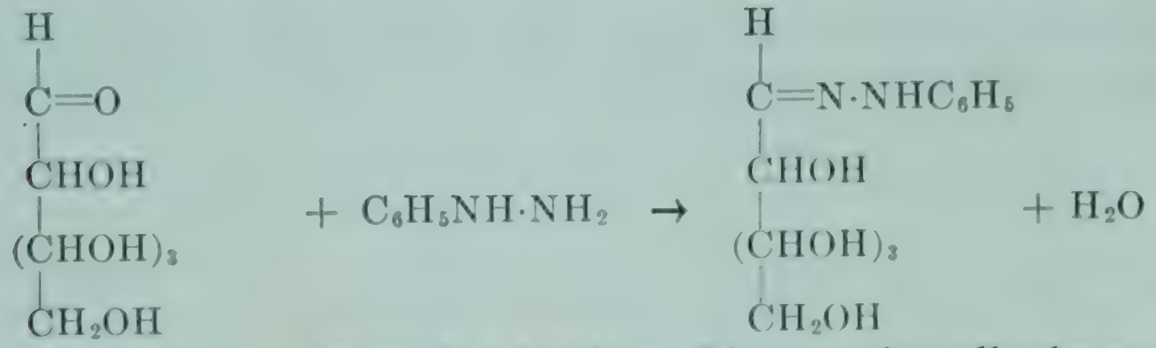
[2] Thymol has the advantage that its solutions do not deteriorate. Like the α-naphthol test it is also given by aldehydes and by acids such as formic, lactic, oxalic, citric, etc., and by acetone. The tests are very delicate, being given by solutions of 0.001 per cent glucose and 0.0001 per cent sucrose.

[3] See Appendix.

The reaction leading to the formation of glucosazone is indicated by the following equations. The intermediate steps between hydrazone and osazone are not known with certainty, despite earlier views to the contrary.

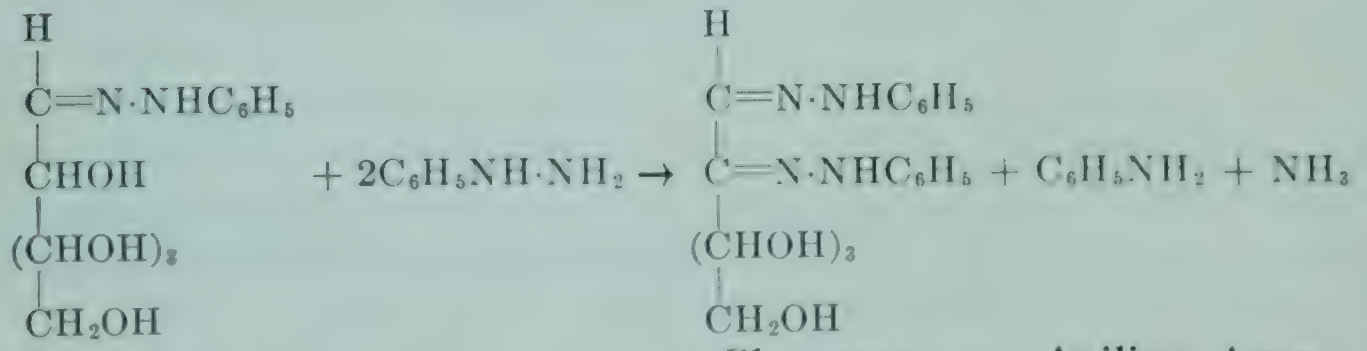
(1)



$$\begin{array}{l}\mathrm{H}\\ \;|\\ \mathrm{C{=}O}\\ \;|\\ \mathrm{CHOH}\\ \;|\\ \mathrm{(CHOH)_3}\\ \;|\\ \mathrm{CH_2OH}\end{array} + \mathrm{C_6H_5NH{\cdot}NH_2} \rightarrow \begin{array}{l}\mathrm{H}\\ \;|\\ \mathrm{C{=}N{\cdot}NHC_6H_5}\\ \;|\\ \mathrm{CHOH}\\ \;|\\ \mathrm{(CHOH)_3}\\ \;|\\ \mathrm{CH_2OH}\end{array} + \mathrm{H_2O}$$

Glucose **Phenylhydrazine** **Glucose phenylhydrazone**

(2)



$$\begin{array}{l}\mathrm{H}\\ \;|\\ \mathrm{C{=}N{\cdot}NHC_6H_5}\\ \;|\\ \mathrm{CHOH}\\ \;|\\ \mathrm{(CHOH)_3}\\ \;|\\ \mathrm{CH_2OH}\end{array} + \mathrm{2C_6H_5NH{\cdot}NH_2} \rightarrow \begin{array}{l}\mathrm{H}\\ \;|\\ \mathrm{C{=}N{\cdot}NHC_6H_5}\\ \;|\\ \mathrm{C{=}N{\cdot}NHC_6H_5}\\ \;|\\ \mathrm{(CHOH)_3}\\ \;|\\ \mathrm{CH_2OH}\end{array} + \mathrm{C_6H_5NH_2} + \mathrm{NH_3}$$

Glucosazone **Aniline** **Ammonia**

4. *Diffusibility of Glucose.* Test the diffusibility of glucose through a membrane, using a dialyzing bag similar to the type described under Experiments on Colloidal Solutions (see p. 10).

All monosaccharides and disaccharides are diffusible, but many polysaccharides are not.

5. Reduction Tests. To their free or potentially free aldehyde or ketone groups many sugars owe in part the property of readily reducing in alkaline solution the ions of certain metals such as copper, bismuth, mercury, iron, and silver. Upon this property of reduction the most widely used tests for sugars are based. For example, when blue cupric hydroxide in suspension in an alkaline liquid is heated it is converted into insoluble black cupric oxide, but if a reducing agent like certain sugars is present, the cupric hydroxide is reduced to insoluble yellow or red cuprous oxide. These changes are indicated as follows:

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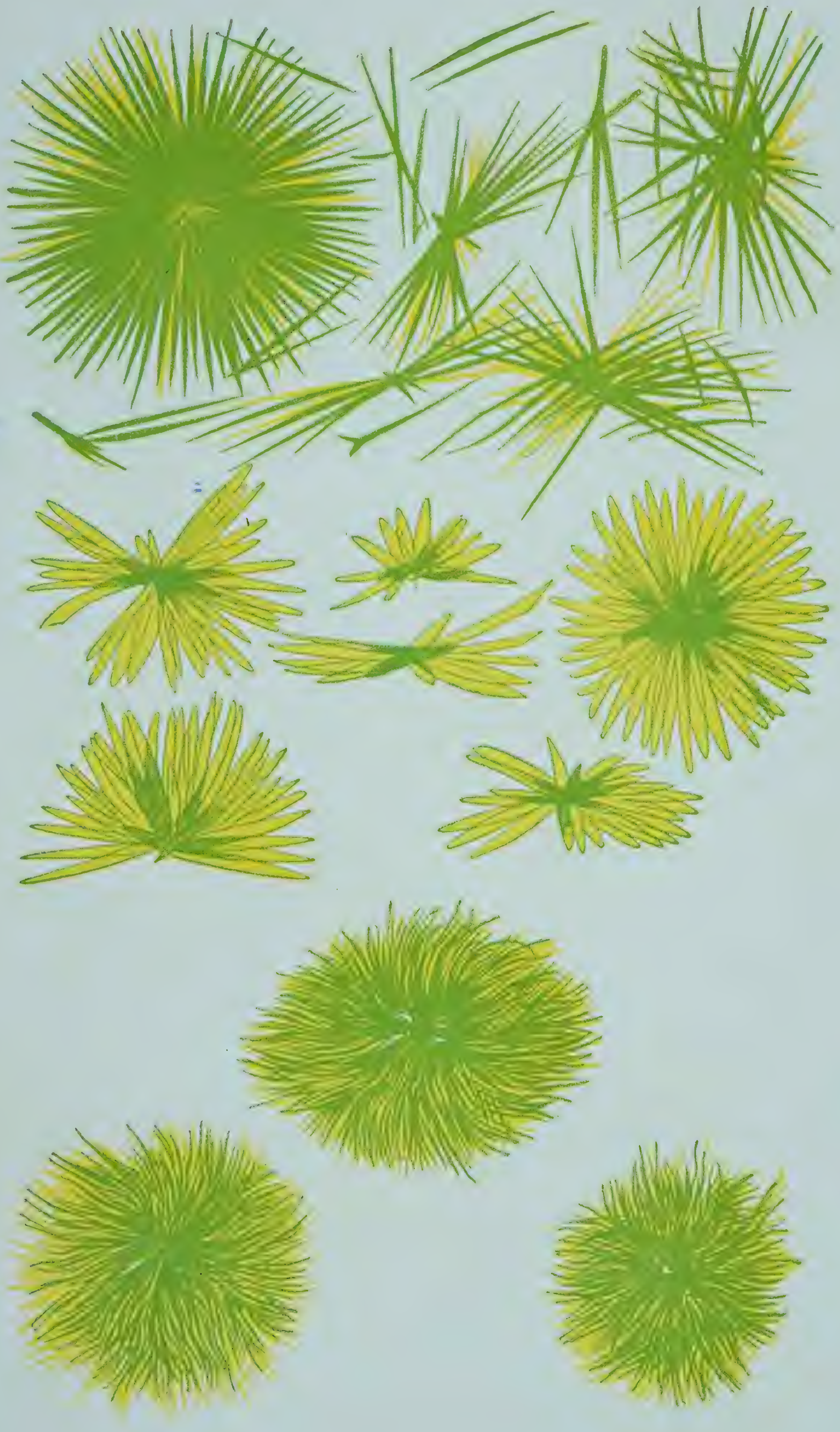
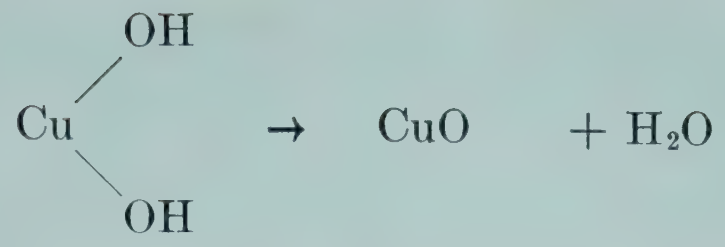
[4] See Moore and Link: *J. Biol. Chem.*, 133, 293 (1940), for a method of obtaining crystalline sugar derivatives claimed to be superior to the osazone reaction.

PLATE II



OSAZONES.

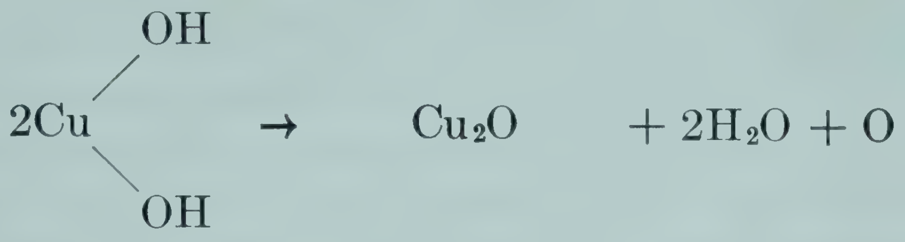
Upper form, dextrosazone (glucosazone); *central form,* maltosazone; *lower form,* lactosazone.



$$\mathrm{Cu}\begin{matrix}\diagup \mathrm{OH} \\ \diagdown \mathrm{OH}\end{matrix} \rightarrow \mathrm{CuO} + \mathrm{H_2O}$$

Cupric hydroxide (blue) Cupric oxide (black)

Reaction in absence of a reducing agent



$$2\mathrm{Cu}\begin{matrix}\diagup \mathrm{OH} \\ \diagdown \mathrm{OH}\end{matrix} \rightarrow \mathrm{Cu_2O} + 2\mathrm{H_2O} + \mathrm{O}$$

Cupric hydroxide Cuprous oxide (yellow to red)

Reaction in presence of a reducing agent

The use of a suspension of a metallic oxide or hydroxide as a reagent is obviously impractical. Certain organic compounds, particularly those containing one or more alcoholic OH groups in the molecule (e.g., tartaric acid, citric acid, glycerol, even the sugars themselves), react in alkaline solution with metallic hydroxides to form a soluble complex ion which, though relatively little ionized, nevertheless dissociates to yield sufficient ions of the metal for reduction reactions to occur. The formation of such a complex is the basis of most of the heavy-metal reagents for reducing sugars.

The alkali in these reagents brings about considerable decomposition of the sugar molecule into reactive fragments which may also reduce the metal ions. Thus while the total reduction for a given concentration of sugar may be constant under carefully defined conditions and is therefore utilizable for quantitative purposes, it is impossible to write a balanced equation for the reaction in terms of the simple oxidation of the sugar and reduction of the metal ion.

The chemical reactions here discussed are exemplified in the following tests. (For the application of these and other tests to the detection of sugar in urine, see Chapter 29.)

a. Fehling's Test. **To about 1 ml. of Fehling's solution[5] in a test tube add about 4 ml. of water, and boil. This is done to determine whether the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. To the warm Fehling's solution add sugar solution a few drops at a time and heat the mixture after each addition. The production of yellow or brownish-red cuprous oxide indicates that reduction has taken place. The differences in color of the cuprous oxide precipitates under different conditions are apparently due to differences in the size of the particles, the more finely divided precipitates having a yellow color while the coarser ones are red. In the presence of protective colloidal substances the yellow precipitate is usually formed.**

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[5] See Appendix.

In testing a solution preserved by chloroform a positive reaction may be obtained in the absence of sugar. This is due to the fact that the hot alkali produces reducing substances from the chloroform.

Ammonium salts also interfere with Fehling's test. If present in excess the solution (e.g., urine) should be made alkaline with Na_2CO_3 and boiled in order to decompose the ammonium salts. Prolonged contact with hot strong alkali may lead to destruction of the sugar present.

If the solution under examination by Fehling's test is acid in reaction it must be neutralized or made alkaline before applying the test.

b. Benedict's Test. **Benedict modified the Fehling solution to produce an improved reagent which has largely displaced the latter in routine laboratory practice. The following is the procedure for the detection of glucose in solution. To 5 ml. of the reagent[6] in a test tube add exactly 8 drops of the solution under examination. Mix well. Boil the mixture vigorously for two minutes (or place in boiling water for three minutes), and then allow the fluid to cool spontaneously (do not hasten cooling by immersion in cold water). In the presence of dextrose the entire body of the solution will be filled with a precipitate which may be red, yellow, or green in color, depending upon the amount of sugar present. In the presence of over 0.2 to 0.3 per cent of glucose, the precipitate will form quickly. If no glucose is present, the solution will remain perfectly clear.**

Even very small quantities of glucose (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for glucose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since *amount* rather than color of the precipitate is made the basis of this test, it may be applied, even for the detection of small quantities of glucose, as readily in artificial light as in daylight. Chloroform does not interfere with this test nor do uric acid or creatinine interfere to such an extent as in the case of Fehling's test.

c. Bismuth Reduction Test (Nylander). **To 5 ml. of sugar solution in a test tube add one-tenth its volume of Nylander's reagent[6] and heat for five minutes in a boiling water bath. The solution will darken if reducing sugar is present, and upon standing for a few moments a black color will appear.**

This color is due to the precipitation of metallic bismuth. If the test is made on a solution containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Glucose when present to the extent of 0.08 per cent may be easily detected by this reaction. Uric acid and creatinine which interfere with the Fehling's test do not interfere with the Nylander test. It is claimed that the bismuth reduction tests give a negative reaction with solutions containing sugar when mercuric chloride or chloroform is present. The inhibitory action of mercuric chloride is questionable; the inhibitory influence of chloroform may be overcome by raising the temperature of the urine to the boiling point for a period of five minutes previous to making the test.

A positive bismuth reduction test is probably due to the following reactions:

---

[6] See Appendix.

$$(a)\quad Bi(OH)_2NO_3 + KOH \rightarrow Bi(OH)_3 + KNO_3$$
$$(b)\quad 2Bi(OH)_3 - 3O \rightarrow 2Bi + 3H_2O$$

d. Barfoed's Test. **To 5 ml. of Barfoed's solution[6] in a test tube, add 0.5 ml. of glucose solution and heat to boiling. Reduction is indicated by the formation of a red precipitate of cuprous oxide. If the precipitate does not appear after boiling for 30 seconds, allow the tube to stand for about 15 minutes and examine.**

To compare reactions of mono- and disaccharides, place 0.5-ml. portions of glucose, fructose, maltose, lactose, and sucrose solutions in each of five test tubes, add 5 ml. of Barfoed's solution to each tube, mix, and place in a boiling water bath. Note the time when signs of reduction first appear in each tube. Continue boiling for 15 minutes, remove the tubes from the bath, and note the amounts of precipitate in the bottom of each tube after it has been standing for 15 minutes. Record your observations.

Barfoed's test is *not* a specific test for glucose, serving simply to detect *monosaccharides.* Disaccharides will also respond to the test under proper conditions of acidity. Also if the sugar solution is boiled in contact with the reagent long enough to hydrolyze the disaccharide through the action of the acetic acid present in the Barfoed's solution, a positive test results. Barfoed's is a copper reduction test, but differs from Fehling's and other reduction tests in that the reduction is brought about in an acid solution. It is unsuited for the detection of sugar in urine or in any fluid containing chlorides.

e. Tauber and Kleiner Modification of Barfoed's Test. **Introduce into one test tube 1 ml. of an approximately 0.1 per cent solution of the sugar to be tested. Put into another tube 1 ml. of water. Add 1 ml. of copper reagent[7] to each. Heat in a boiling water bath for 3 minutes, cool for 2 minutes. Add 1 ml. of color reagent[8] to each. Mix. A blue color will be obtained if monosaccharides are present. With only disaccharides present the color will be the same as in the control. Chlorides interfere but not in amounts as large as 5 mg. per ml. of solution.**

f. Picric-Acid Test. **To 5 ml. of the sugar solution add 2 to 3 ml. of saturated picric acid solution and about 1 ml. of 10 per cent Na_2CO_3. Warm. Note the development of a mahogany-red color in the presence of glucose due to reduction of the picric acid with the formation of picramic acid:**

$$\underset{\text{Picric acid}}{C_6H_2{\cdot}OH(NO_2)_3} \rightarrow \underset{\text{Picramic acid}}{C_6H_2{\cdot}OH{\cdot}NH_2(NO_2)_2}$$

6. Alcoholic Fermentation. **Prepare 500 ml. of a concentrated (10 per cent) solution of glucose, add a small amount of egg albumin or commercial peptone, and introduce the mixture into a liter flask. Add yeast, and by means of a bent tube connect this flask with a second flask containing a solution of barium hydroxide protected from the air by a soda-lime tube in the stopper (Fig. 13). Place the flasks in a warm place and note the passage of gas bubbles into the barium hydroxide solution. As these gas bubbles (CO_2) enter, a white precipitate of barium carbonate will form. The glucose has been fermented**

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[7] See Appendix.

[8] Benedict's or Folin's phosphomolybdic reagents for sugar in blood may be used. See blood-sugar methods, Chapter 23.

according to the following equation:

$$C_6H_{12}O_6 \rightarrow 2C_2H_5OH + 2CO_2$$

The important intermediate products formed during this reaction are indicated below. (For further details see Chapter 33 on carbohydrate metabolism.)

Hexose → Hexose di-phosphoric acid → Phosphoglyceric acid → Pyruvic acid → Acetaldehyde → Ethyl alcohol

When the activity of the yeast has practically ceased, decant the supernatant fluid, return it to the cleaned flask, connect with a condenser, and distil. Catch the first portion of the distillate, which may be redistilled if its alcohol content is low, and test for alcohol by the following reaction:

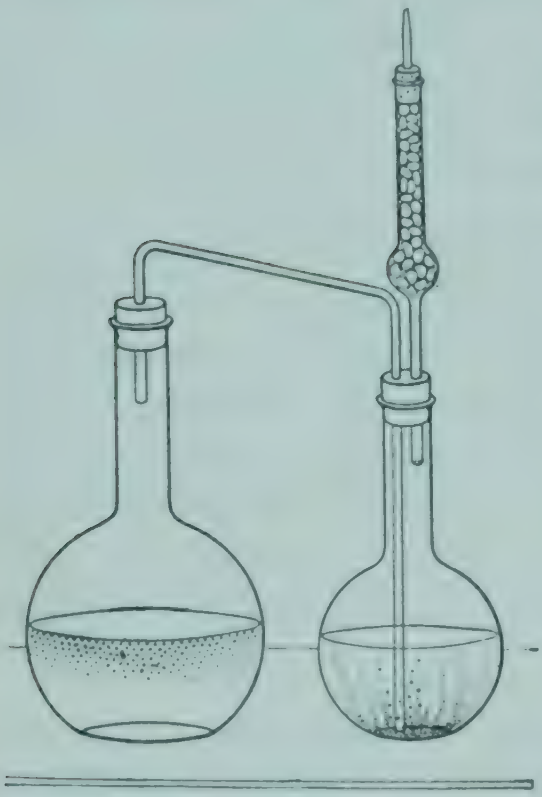

FIG. 13. FERMENTATION APPARATUS.

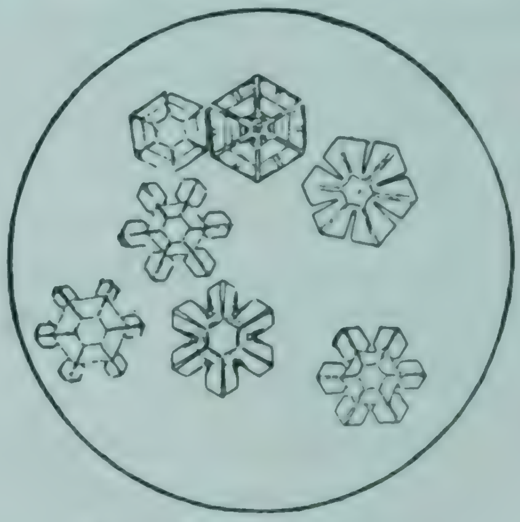

FIG. 14. IODOFORM.

(Autenrieth)

Iodoform Test. Render 2 to 3 ml. of the distillate alkaline with sodium hydroxide solution and add a few drops of strong iodine solution—e.g., Lugol's. Warm gently and note the formation of iodoform crystals.[9] Examine these crystals under the microscope and compare them with those in Fig. 14.

7. *Fermentation Test.* "Rub up" in a mortar about 20 ml. of the sugar solution with a small piece of compressed yeast. Transfer the mixture to a saccharometer (Fig. 15) and put it aside in a warm place for about 12 hours. If the sugar is fermentable, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermentation introduce 2 to 3 ml. of 10 per cent sodium hydroxide solution into the graduated portion by means of a bent pipet, fill the bulb portion with water, place the thumb tightly over the opening in the apparatus, and invert the saccharometer. Remembering that NaOH has the power to absorb CO_2, how do you explain the result?[10] Filter some of the mixture. To 5 ml. of

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[9] The formation of iodoform may be represented by the following equations:

$$\begin{array}{ll} CH_3{\cdot}CH_2OH + I_2 + 2NaOH & \rightarrow CH_3{\cdot}CHO + 2NaI + 2H_2O \\ CH_3{\cdot}CHO + 3I + 3NaOH & \rightarrow CI_3{\cdot}CHO + 2NaI + 3H_2O \\ CI_3{\cdot}CHO + NaOH & \rightarrow CHI_3 + HCOONa \end{array}$$

[10] The liberation of carbon dioxide by yeast is not necessarily a criterion of the presence of sugar. The presence of an enzyme, called *carboxylase*, has been demonstrated in yeast, which has the power of splitting off CO_2 from the carboxyl group of amino- and other aliphatic acids.

the filtrate add several drops (enough to give a yellow color to the whole mixture), of a strong solution of iodine in potassium iodide (e.g., Lugol's). Warm gently. Note the iodoform odor and examine under a microscope for crystals of iodoform. What does a positive test here indicate?

***8. Demonstration of Optical Activity.* A demonstration of the use of the polariscope should be made by the instructor, each student being required to take readings and apply the polariscope to the determination of either the specific rotation or the concentration of a sugar.**

THE POLARISCOPE

For a detailed description of the different forms of polariscopes, the method of manipulation, and the principles involved, the student is referred to any standard textbook of physics. A brief description follows.

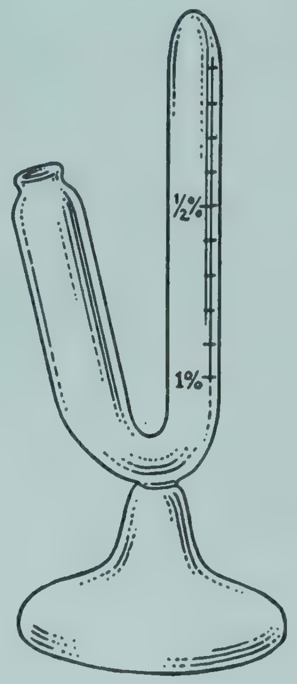

FIG. 15. EINHORN SACCHAROMETER.

Waves of ordinary light vibrate in all planes perpendicular to the direction of propagation. By certain means light may be caused to vibrate in but a single plane, and is then said to be plane polarized. Thus if a ray of light is passed through a crystal of calcite (natural crystallized calcium carbonate), it is divided into two rays (each polarized) vibrating in planes perpendicular to each other (Fig. 16). As the two rays are unequally bent it is possible to completely separate them and thus obtain light vibrating in but a single plane. For this purpose we may use a Nicol prism consisting of two pieces of calcite cemented together with Canada balsam. The ray of light *l* on entering the prism is divided into two rays. One, called the ordinary ray *o*, is reflected from the Canada balsam to the exterior and absorbed by the black varnish coating the prism. The other, the extraordinary ray *e*, passes through the balsam and emerges from the prism as polarized light. Many organic substances (sugar, proteins, etc.) have the power of twisting, or rotating, this plane of polarized light, the extent to which the plane is rotated depending upon the nature of the molecule and upon the number of molecules which the polarized light passes. Substances which possess this power are said to be *optically active.* The *specific rotation* of a substance is the rotation expressed in degrees which is afforded by 1 g. of substance dissolved in 1 ml. of water in a tube 1 decimeter in length. The specific rotation, $(\alpha)_D$, may be calculated by means of the following formula:

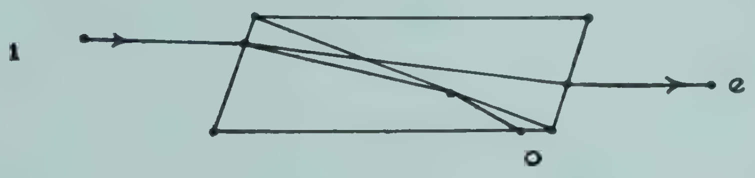

FIG. 16. PATH OF LIGHT RAY THROUGH NICOL PRISM.

$$(\alpha)_D = \frac{\alpha}{p \cdot l}$$

in which

$_D$ = sodium light,
α = observed rotation in degrees,
p = g. of substance dissolved in 1 ml. of liquid,
l = length of the tube in decimeters.

If the specific rotation has been determined and it is desired to ascertain the percentage of the substance in solution, this may be obtained by the use of the following formula:

$$p = \frac{\alpha}{(\alpha)_D l}$$

The value of p multiplied by 100 will be the percentage of the substance in solution.

SPECIFIC ROTATIONS OF MORE COMMON CARBOHYDRATES[11]

D-Glucose	+ 52.5°	Sucrose	+ 66.5°
D-Fructose	− 92.3°	Lactose	+ 52.5°
D-Galactose	+ 81.5°	Maltose	+137.0°
D-Mannose	+ 14.2°	Raffinose	+104.0°
L-Arabinose	+104.5°	Dextrin	+195.0°
D-Xylose	+ 19.0°	Starch (soluble)	+196.0°
Rhamnose	+ 9.0°	Glycogen	+197.0°

An instrument by means of which the extent of the rotation may be determined is called a polariscope or polarimeter. Such an instrument designed especially for the examination of sugar solutions is termed a saccharimeter or polarizing saccharimeter. The form of polariscope in Fig. 17 consists essentially of a long barrel provided with a Nicol prism at either end (Fig. 18). The solution under examination is contained in a tube which is placed between these two prisms. At the front end of the instrument is an adjusting eyepiece for focusing and a large recording disk which registers in degrees and fractions of a degree. The light is admitted into the far end of the instrument and is polarized by passing through a Nicol prism. This polarized ray then traverses the column of liquid within the tube mentioned above and if the substance is optically active the plane of the polarized ray is rotated to the right or left. Compounds rotating the ray to the right are called dextrorotatory and those rotating it to the left levorotatory.

Within the apparatus is a disk which is so arranged as to be without lines and uniformly light at zero. Upon placing the optically active substance in position, however, the plane of polarized light is rotated or turned and it is necessary to rotate the disk through a certain number of degrees in order to secure the normal conditions—i.e., without lines and uniformly light. The difference between this reading and the *zero* is α, or the observed rotation in degrees.

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[11] The specific rotation varies with the temperature and concentration of the solution. The figures here given are for concentrations of about 10 per cent and temperatures of about 20° C. Fresh solutions may give markedly different values due to mutarotation, the figures here given representing the constant values obtained on standing.

Sugar solutions (glucose, levulose, lactose, and maltose, but not sucrose) when freshly prepared possess a changing rotation—so-called mutarotation. For this reason such solutions before polariscopic examination should be allowed to stand over night, heated to 100° C. and then cooled, or treated with a drop of ammonia followed by a drop of acid. This brings about an equilibrium between the α and β forms possessing different rotations, such as those of which ordinary glucose is a mixture.

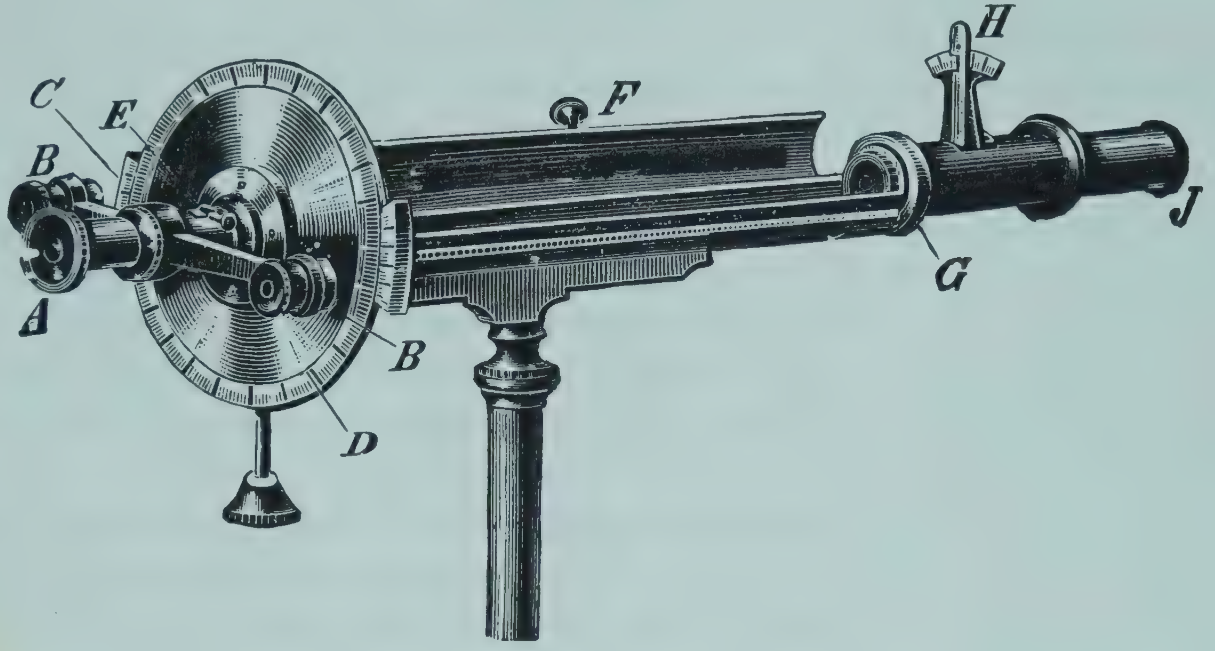


FIG. 17. ONE FORM OF LAURENT POLARISCOPE.

(*B*) Microscope for reading the scale; (*C*) a vernier; (*E*) position of the analyzing Nicol prism; (*H*) polarizing Nicol prism in the tube below this point.

Polarizing saccharimeters are also constructed by which the percentage of sugar in solution is determined by making an observation and multiplying the value of each division on a horizontal sliding scale by the value of the division expressed in terms of dextrose. This factor may vary according to the instrument.

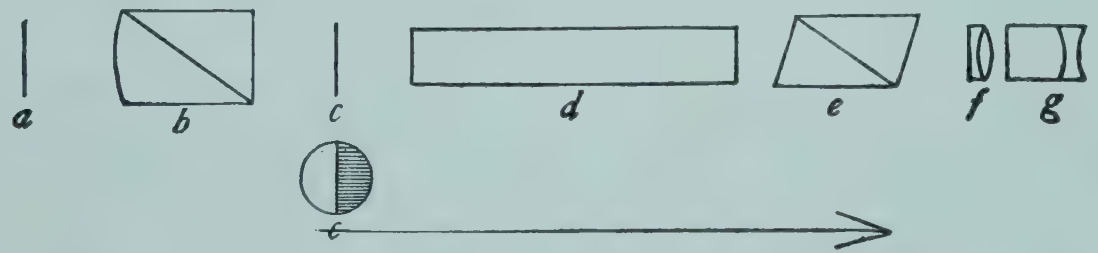
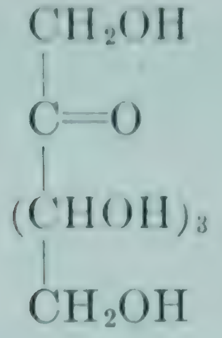


FIG. 18. DIAGRAMMATIC REPRESENTATION OF COURSE OF LIGHT THROUGH LAURENT POLARISCOPE.

The direction is reversed from that of Fig. 17. (*a*) Bichromate plate to purify light; (*b*) polarizing Nicol prism; (*c*) a thin quartz plate covering one-half the field and essential in producing a second polarized plane; (*d*) tube to contain liquid under examination; (*e*) analyzing Nicol prism; (*f*) and (*g*) ocular lenses.

Methods embracing the determination of the optical rotation are utilized in many analytical procedures for identifying liquids or solutions, as well as for establishing the composition of solutions.

FRUCTOSE

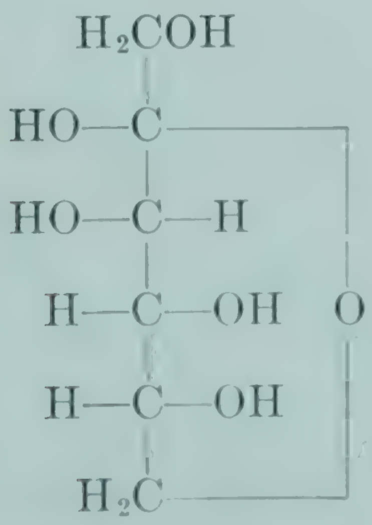


$$\begin{array}{c} CH_2OH \\ | \\ C{=}O \\ | \\ (CHOH)_3 \\ | \\ CH_2OH \end{array}$$

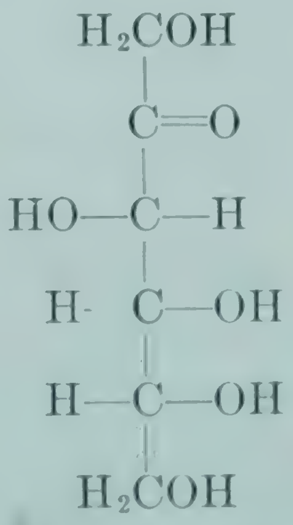
As already stated, fructose, sometimes called levulose or fruit sugar, occurs widely disseminated throughout the plant kingdom in company with glucose. It has been prepared commercially from the inulin of the Jerusalem artichoke. It is not found in detectable amounts in animal tissues or fluids except under certain exceptional conditions, but in the form of its phosphoric acid esters it is a recognized intermediate in carbohydrate metabolism.

Although fructose is a ketose it nevertheless reduces metallic oxides in alkaline solution, owing to the presence in it of the terminal group $CO \cdot CH_2OH$. For the same reason monohydroxyacetone ($CH_3 \cdot CO \cdot CH_2OH$) also reduces such solutions although acetone ($CH_3 \cdot CO \cdot CH_3$) does not. With phenylhydrazine, fructose forms the same osazone as glucose. With methylphenylhydrazine, it forms an osazone more rapidly than does glucose.

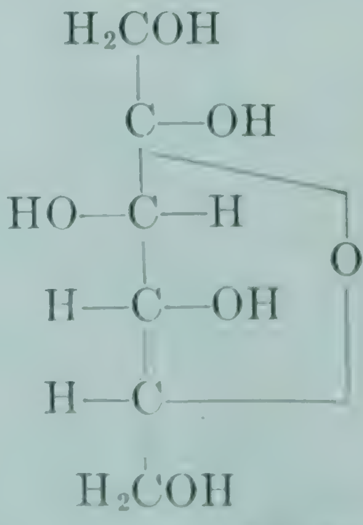
Fructose in solution undergoes mutarotation just as glucose does, and for similar reasons, the rotation of a fresh solution being $-133.5°$ which changes to $-92.3°$ on standing or in the presence of a trace of alkali. The relation between the free ketone form and the α and β forms, and the existence of pyranose and furanose structures, are similar to the situation already described for glucose and are illustrated by the formulas which follow:



β-D-fructopyranose



D-fructose (ketone form)



α-D-fructofuranose

Ordinary fructose consists chiefly of the pyranose form; fructose in the combined state, as in sucrose and inulin, appears to be invariably in the more reactive furanose form.

EXPERIMENTS ON FRUCTOSE

***1–6.* Repeat Solubility, Benedict's, Phenylhydrazine, Barfoed's, Nylander's, and Fermentation tests as given for Glucose.**

7. *Resorcinol-Hydrochloric Acid Reaction* (*Selivanoff*). To 5 ml. of Selivanoff's reagent[12] in a test tube add 5 drops of a fructose solution and heat the mixture to boiling. A positive reaction is indicated by the production of a red color with or without the separation of a brown-red precipitate. The latter may be dissolved in alcohol, to which it will impart a striking red color.

To compare the reactions of aldose and ketose sugars, place 0.5-ml. portions of glucose, fructose, maltose, lactose, and sucrose solutions into each of five test tubes, add 5 ml. of Selivanoff's reagent to each tube, mix, and place in a boiling water bath. Note the time when color first appears in each tube. Continue boiling for 15 minutes, noting the color developed in each tube at approximately 5-minute intervals. Record your observations.

This test is also given by sucrose which is hydrolyzed during the course of the test yielding fructose as one product. If the boiling be prolonged a similar reaction may be obtained with solutions of glucose or maltose. This has been explained in the case of glucose as due to the transformation of the glucose into fructose by the catalytic action of the hydrochloric acid. The precautions necessary for a positive test for levulose are as follows: The concentration of the hydrochloric acid must not be more than 12 per cent. The reaction (red color) and the precipitate must be observed after not more than 20 to 30 seconds' boiling. Glucose must not be present in amounts exceeding 2 per cent. The precipitate must be soluble in alcohol with a bright red color.

8. *Aminoguanidine Reaction* (*Tauber*).[13] Place 0.5 ml. of concentrated sulfuric acid in a test tube. Add 0.2 ml. of a 2.5 per cent aqueous aminoguanidine sulfate solution without mixing. Add 0.2 ml. of the test solution and mix well. In the presence of ketohexoses or compounds which yield ketohexose, a bright reddish-purple color is formed in about 1 minute and persists for several hours.

As little as 0.05 mg. of fructose is said to be detectable by this test. Sucrose and inulin also give a positive test. Aldohexose up to a concentration of 1 per cent does not interfere. Higher concentrations give a positive reaction, so solutions to be tested should be adjusted by dilution to contain not more than 0.5 – 1.0 per cent total carbohydrate. No color is given by aldopentoses, starch, glycogen, various pure proteins, or formaldehyde.

9. *Formation of Methylphenylfructosazone.* To a solution of 1.8 g. of levulose in 10 ml. of water add 4 g. of methylphenylhydrazine and enough alcohol to clarify the solution. Introduce 4 ml. of 50 per cent acetic acid and heat the mixture for 5 to 10 minutes (no longer) on a boiling water bath. On standing 15 minutes at room temperature, crystallization begins and is complete in two hours. By scratching the sides of the flask or by inoculation, the solution quickly congeals to form a thick paste of reddish-yellow silky needles. These are the crystals of methylphenylfructosazone. They may be recrystallized from hot 95 per cent alcohol (m. p. 153° C.). Glucose may give the same osazone more slowly (after five hours).

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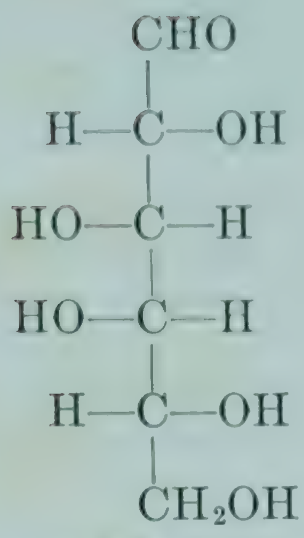
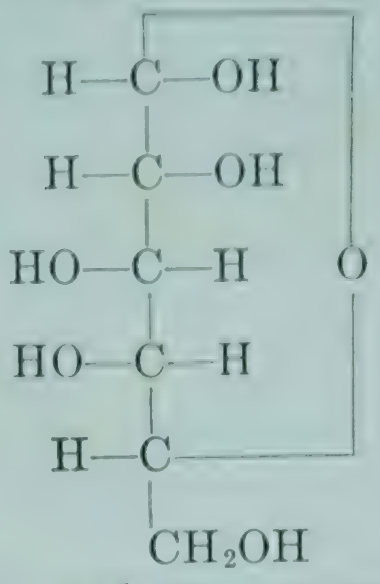
[12] See Appendix.

[13] Tauber: *J. Biol. Chem.*, **182**, 605 (1950).

GALACTOSE



D-galactose



α-D-galactopyranose

Galactose occurs with glucose as one of the products of the hydrolysis of lactose. It is also found as a constituent of the galactolipides of nervous tissue. Galactose is a typical aldohexose, is dextrorotatory and exhibits mutarotation in solution, and forms a characteristic osazone with phenylhydrazine. It ferments either very slowly or not at all with ordinary yeast, although some varieties of yeast ferment it readily. Upon oxidation with nitric acid, galactose yields mucic acid (Fig. 19), thus differentiating this monosaccharide from glucose and fructose. Lactose also yields mucic acid under these conditions. Mucic acid is $COOH(CHOH)_4COOH$, the H and OH groups on carbon atoms 2 to 5 having the same spatial configuration as for galactose itself. Although mucic acid contains four asymmetric carbon atoms, it is optically inactive because one half of the molecule is the mirror image of the other half (so-called *internal compensation*).

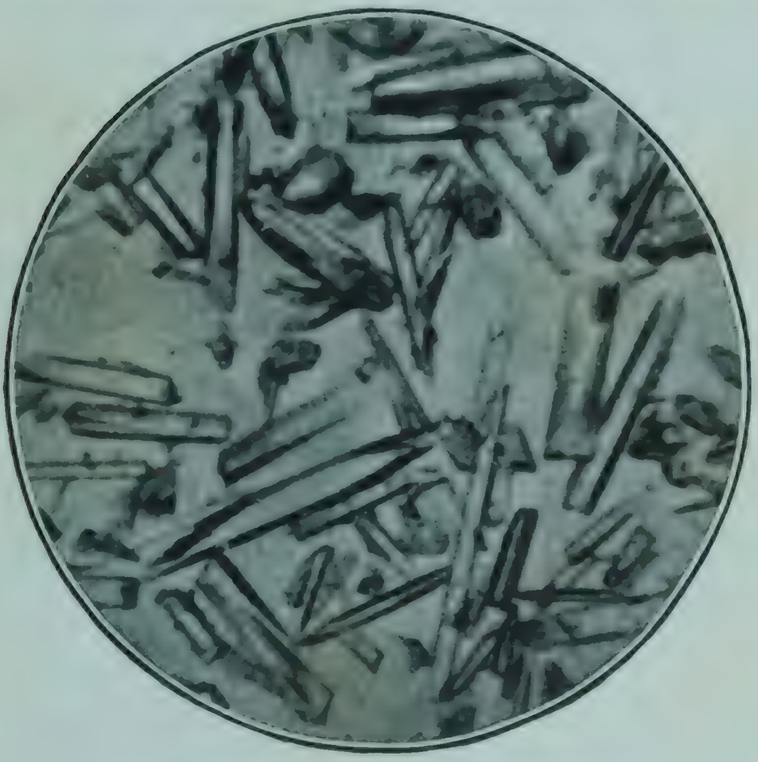


FIG. 19. MUCIC ACID CRYSTALS.

From a photomicrograph furnished by Prof. William H. Welker.

EXPERIMENTS ON GALACTOSE

1. *Phloroglucinol—Hydrochloric Acid Reaction* (Tollens). To equal volumes of galactose solution and hydrochloric acid (sp. gr. 1.19) add a little phloroglucinol, and heat the mixture on a boiling water bath. Galactose, pentose, and glycuronic acid will be indicated by the appearance of a red color. Galactose may be differentiated from the two latter substances in that its solutions exhibit no absorption bands upon spectroscopic examination.

2. *Mucic Acid Test.*[14] Place about 50 mg. of galactose in a clean, dry test tube, add 1 ml. of distilled water and 1 ml. of concentrated nitric acid. Heat in a

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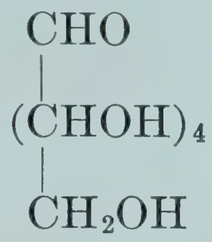
[14] This modification of the mucic acid test was suggested by Dr. A. G. Cole of the Uni-

boiling water bath for 1½ hours. Let stand over night. A crystalline precipitate of mucic acid, which may be identified by microscopical examination of the crystals, forms under these conditions.

3. Phenylhydrazine Reaction. **Make the test according to directions given for Glucose (p. 63).**

MANNOSE



$$\begin{array}{l} CHO \\ | \\ (CHOH)_4 \\ | \\ CH_2OH \end{array}$$

Mannose is an aldohexose, differing structurally from glucose solely in the spatial arrangement of H and OH groups around carbon atom number 2 (see structure, p. 58). It is found in nature chiefly in the form of polysaccharides called mannans or mannosans which occur in plants, particularly in certain seeds ("vegetable ivory"), and from which mannose may be obtained by hydrolysis. Interest in mannose has been heightened by the discovery that it is present in small amount in certain animal proteins.[15] Mannose is a reducing sugar and is fermentable by yeast. It is readily distinguished from glucose by the formation of a sparingly soluble, colorless, crystalline phenylhydrazone when treated with phenylhydrazine at room temperature.

EXPERIMENTS ON MANNOSE

1–4. **Repeat Benedict's, Barfoed's, Selivanoff's, and Fermentation tests as given for Glucose and Fructose (pp. 62, 72).**

5. Reaction with Phenylhydrazine. **Place a small amount of solid phenylhydrazine reagent in a test tube and add 5 ml. of mannose solution. Shake well and allow to stand at room temperature for 10 or 15 minutes. Observe the development of a colorless crystalline precipitate of mannose phenylhydrazone (examine a drop under the microscope). When the hydrazone has been obtained, place the tube in boiling water for one-half to three-quarters of an hour, remove, and allow to cool slowly. What change has occurred? Examine a drop of the suspension at this point under the microscope. Explain. As a control, a glucose solution may be carried through the same experimental procedure.**

PENTOSES, $C_5H_{10}O_5$

Pentoses are usually defined as sugars containing five carbon atoms in the molecule, although rhamnose, $C_6H_{12}O_5$, a methylpentose, is an exception to this statement. The pentoses are widely distributed in plant and animal tissues, usually as components of some larger molecule. In plants, and more particularly in certain gums, pentoses occur as complex poly-

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versity of Illinois College of Medicine. Smaller quantities of galactose (or lactose) may be used (20 mg.), but in such cases crystallization may take two or three days.

[15] Rimington: *Biochem. J.*, **23**, 430 (1929); Sørensen and Hangard: *Compt. rend. trav. lab. Carlsberg*, **19**, No. 12 (1933).

saccharides called pentosans, from which the free pentose (e.g., arabinose, xylose) may be obtained on acid hydrolysis. In both plant and animal tissues certain pentoses (ribose, deoxyribose) are universally found as constituents of the nucleoproteins of the cell, being present in the nucleic acid portion of the molecule. Ribose is likewise an essential component of certain mono- and dinucleotides found in cells, such as adenylic acid, coenzymes I and II, and riboflavin (vitamin B_2).

As a class the pentoses may be either aldoses or ketoses, are nonfermentable by yeast, have strong reducing power, and form osazones with phenylhydrazine. The stereochemistry of the pentoses is similar to that already described for the hexoses. The pyranose ring appears to be the most common for the free sugars. In nucleic acids, ribose is present in the furanose form. For structures of the various aldopentoses, see p. 58. On distillation with strong hydrochloric acid, pentoses and pentosans yield furfural, a reaction which is used not only for the quantitative determination of pentoses but also in the commercial production of furfural from plant by-products such as oat hulls.

Pentoses are an important constituent of the diet of herbivorous animals. Their role in human nutrition is not well established. In the rare and apparently harmless condition known as pentosuria (p. 844) significant amounts of the pentose xyloketose are found in the urine and may lead to a false diagnosis of diabetes mellitus. Pentosuria is also said to occur in normal individuals after the ingestion of large amounts of certain fruits.

The following experiments on pentoses may be carried out on L(+)-arabinose as a typical aldopentose. The L-arabinose may be obtained from gum arabic or from plum or cherry gum by boiling for 10 minutes with concentrated hydrochloric acid.

EXPERIMENTS ON PENTOSES

1. *Benzidine Reaction (Tauber)*.[16] To 0.5 ml. of a 4 per cent solution of benzidine in glacial acetic acid in a test tube add 1 drop of pentose solution. Heat to boiling for several minutes and then cool immediately in cold water. The presence of pentose is indicated by the immediate appearance of a pink to red color. It is said that aldopentoses, but not ketopentoses, respond to this test.

In the authors' experience, this is much the best test for aldopentoses (for other tests and the application to urine, see p. 845. The benzidine reagent is stable for about four days. The common hexoses do not interfere. Substances containing pentose in the molecule, such as the various nucleotides, will give a positive test, but pentosans do not because they are not hydrolyzed under the conditions of the test.

2. *Phenylhydrazine Reaction*. Make this test on the pentose solution as described for glucose. For illustration of pentosazone crystals, see p. 844.

GLYCOSIDES

Practically all the carbohydrates or carbohydrate-containing compounds found in nature which are more complex than the simple sugars just described may be considered to be derivatives of these sugars of the type known generally as glycosides. If the sugar is glucose, the compound is known specifically as a glucoside; if galactose, a galactoside, and so on. The di-, tri-, and polysaccharides are glycosides, as well as a wide variety of naturally occurring substances such as phlorizin, salicin, amygdalin, digitalin, etc., which contain in addition to a sugar residue a specific non-sugar portion which is known as the *aglycone.*

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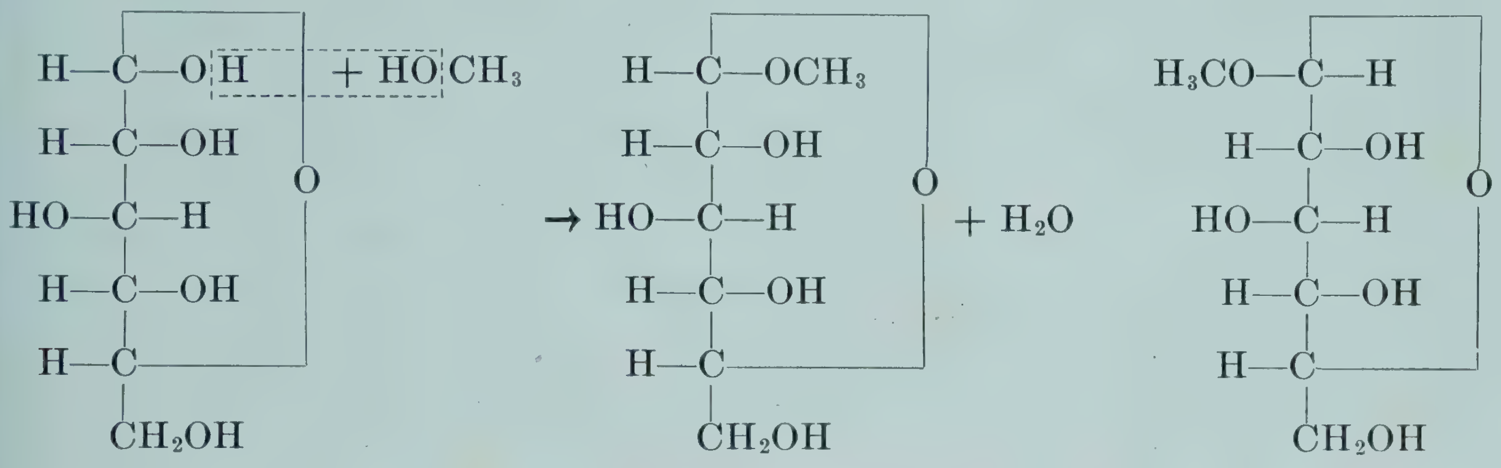
[16] Tauber: *Proc. Soc. Exptl. Biol. Med.*, 37, 600 (1937).

Glycoside formation may be illustrated by the reaction between glucose and methyl alcohol, in which under the proper conditions a molecule of water is split off between an OH group in the glucose molecule and the OH of the methyl alcohol, to give a methyl glucoside:



α-D-glucose **Methyl α-D-glucoside** **Methyl β-D-glucoside**

Since the reaction involves the OH group on carbon number 1 of the glucose molecule, two methyl glucosides are possible as shown, corresponding to the α and β forms of the glucose molecule, and in general both α and β glycosides are found in nature. They may be readily distinguished by the use of enzymes which catalyze the hydrolytic splitting of either the α or β glycoside linkage specifically. For example, in the presence of the enzyme maltase, only α glycosides are hydrolyzed, whereas the enzyme emulsin behaves similarly with respect to β glycosides. In the presence of acids both types of glycosides are readily hydrolyzed to yield their component molecules.

DISACCHARIDES

The disaccharides may be regarded as glycosides in which both components of the molecule are sugars. The common disaccharides have the general formula $C_{12}H_{22}O_{11}$, and yield hexoses on hydrolysis, a molecule of water being taken up in the reaction:

$$C_{12}H_{22}O_{11} + H_2O = 2C_6H_{12}O_6$$

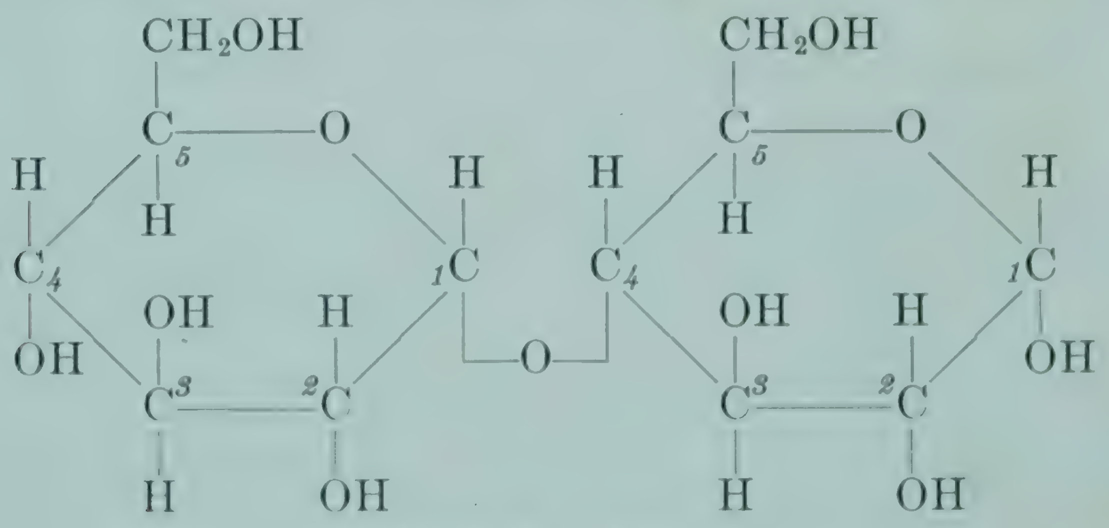
The products of hydrolysis of the more common disaccharides are as follows:

Maltose = glucose + glucose
Lactose = glucose + galactose
Sucrose = glucose + fructose

In the formation of a glycoside linkage between two hexoses, the reducing property of one hexose is ordinarily lost. If the reducing property of the second hexose is not involved the resulting disaccharide is a reducing sugar and exhibits the general properties of such a substance (e.g., osazone formation, mutarotation, etc.). Maltose and lactose are examples of reducing disaccharides. In sucrose, however, the glycoside linkage involves the potential reducing group of both the glucose and fructose components. Sucrose therefore is not a reducing sugar, does not form osazones, and does not show mutarotation in solution.

MALTOSE, $C_{12}H_{22}O_{11}$

Maltose or malt sugar is formed in the hydrolysis of starch through the action of an enzyme, vegetable amylase (diastase), contained in sprouting barley or malt. Certain enzymes in the saliva and in the pancreatic juice cause a similar hydrolysis. Maltose is also an intermediate product of the action of dilute mineral acids upon starch. It is strongly dextrorotatory, shows mutarotation, reduces metallic ions in alkaline solution, and is fermentable by yeast after being converted to glucose by the enzyme maltase of the yeast. In common with the other disaccharides maltose may be hydrolyzed by dilute acid with the formation of two molecules of monosaccharide. In this instance the products are two molecules of glucose. With phenylhydrazine maltose forms an osazone, maltosazone. The following formula represents the probable structure of maltose.



Maltose (α form)
D-glucose 4-(α-D-glucoside)

As its chemical name indicates, maltose is a glucose α-glucoside with a 1,4 linkage. Other disaccharides yielding only glucose on hydrolysis are known, differing from maltose in the type and position of the glucoside bond. Thus cellobiose, a glucose β-glucoside with a 1,4 linkage, is formed during the partial hydrolysis of cellulose. Gentiobiose, a rare disaccharide obtained from the roots of *Gentiana lutea*, is a glucose β-glucoside with a 1,6 linkage. Trehalose, obtained from yeast, is a nonreducing glucose α-glucoside with a 1,1 linkage. Isomaltose, found as a minor end product of the action of amylases upon starch, is a glucose α-glucoside with a 1,6 linkage.

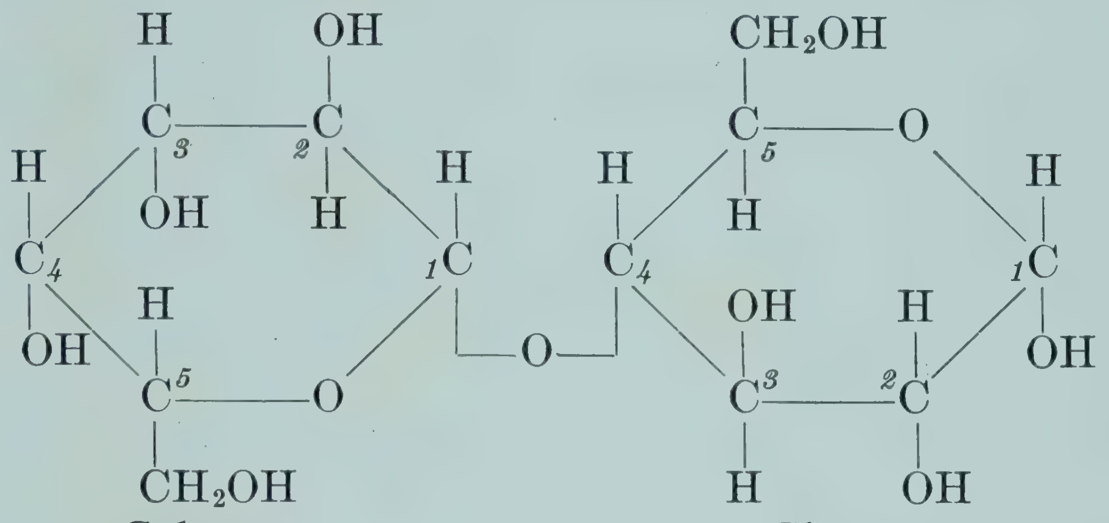
EXPERIMENTS ON MALTOSE

1-6. **Repeat Solubility, Benedict's, Nylander's, Phenylhydrazine, Barfoed's, and Fermentation tests as given for Glucose, pp. 62 ff.**

LACTOSE, $C_{12}H_{22}O_{11}$

Lactose or milk sugar occurs ordinarily only in milk, but it has often been found in the urine of women during the period of lactation.

Lactose is a reducing disaccharide, is dextrorotatory, exhibits mutarotation in solution, and forms an osazone with phenylhydrazine. On hydrolysis it yields glucose and galactose. Chemical evidence indicates that the glycoside linkage involves carbon number 1 of the galactose molecule; lactose is therefore a galactoside. Enzymatic studies indicate that the galactoside linkage has the β configuration. The structure of lactose is probably as follows:



Lactose (α form)
D-glucose 4-(β-D-galactoside)

Since lactose exhibits mutarotation, it exists in α and β forms. The α form is the commonly obtained variety although the β form has become commercially available. It is more soluble than the α form and has been recommended for infant feeding.

Lactose is *not* fermentable by ordinary bakers' yeast. Thus when glucose and lactose are present together in solution they may be differentiated in terms of the reducing power of the solution before and after fermentation. On oxidation with nitric acid lactose yields the sparingly soluble mucic acid because of the presence of galactose in the molecule. This reaction may be used to identify lactose under the proper conditions.

In the souring of milk the *Lactobacillus acidophilus* or *Streptococcus lacticus* and certain other microorganisms bring about lactic acid fermentation by transforming the lactose of the milk into lactic acid CH_3-CHOH·COOH. This same reaction may occur in the alimentary canal as the result of the action of *L. acidophilus* and certain other organisms. In the preparation of kefir and koumiss the lactose of the milk undergoes alcoholic fermentation, through the action of ferments other than yeast, and at the same time lactic acid is produced.

EXPERIMENTS ON LACTOSE

***1–6.* Repeat Solubility, Benedict's, Phenylhydrazine, Barfoed's, Nylander's, and Fermentation tests as given for Glucose, pp. 62 ff.**

7. *Mucic Acid Test.* Repeat test as given for Galactose, p. 74.

SUCROSE, $C_{12}H_{22}O_{11}$

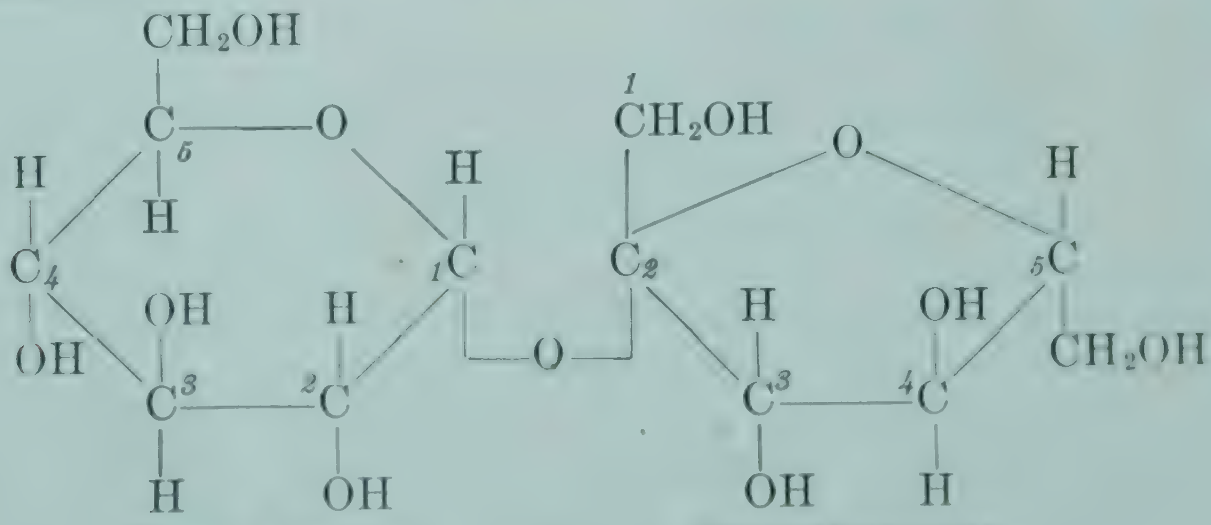
Sucrose, also called saccharose or cane sugar, is one of the most important of the sugars and occurs very extensively distributed in plants, particularly in the sugar cane, sugar beet, sugar millet, and certain palms and maples.

Sucrose is dextrorotatory, and, as before mentioned, upon hydrolysis the molecule of sucrose takes on a molecule of water and breaks down into two molecules of monosaccharide. The monosaccharides formed in this instance are glucose and fructose. This is the reaction:

$$\underset{\textbf{Sucrose}}{C_{12}H_{22}O_{11}} + H_2O \rightarrow \underset{\textbf{Glucose}}{C_6H_{12}O_6} + \underset{\textbf{Fructose}}{C_6H_{12}O_6}$$

This hydrolysis may be produced by bacteria, enzymes, and certain weak acids. After hydrolysis the previously strongly dextrorotatory solution becomes levorotatory. This is due to the fact that the fructose molecule is more strongly levorotatory than the glucose molecule is dextrorotatory. The reaction is therefore frequently called inversion, and the mixture of glucose and fructose obtained is called invert sugar.

Sucrose does *not* reduce metallic ions in alkaline solution and forms *no* osazone with phenylhydrazine. Prolonged boiling in the presence of an *acid* phenylhydrazine solution will, however, hydrolyze the sucrose and cause the formation of glucosazone. It is not fermentable directly by yeast, but must first be inverted by the enzyme sucrase (invertase or invertin) contained in the yeast. The probable structure of sucrose may by represented by the following formula. (Note the absence of any potential ketone or aldehyde group.) Upon inversion the active form of fructose indicated in the formula is rapidly transformed to the more stable modification.



Glucopyranose **Fructofuranose**

Sucrose

α-D-glucopyranosyl-β-D-fructofuranoside

EXPERIMENTS ON SUCROSE

1–7. Repeat Solubility, Benedict's, Nylander's, Barfoed's, Phenylhydrazine, Selivanoff's, and Fermentation tests according to the directions given for Glucose, pp. 62 ff.

8. Inversion of Sucrose. To 25 ml. of sucrose solution in a beaker add 5 drops of concentrated H_2SO_4 and boil one minute. Cool the solution and render

neutral with saturated barium hydroxide. Filter off the precipitate of barium sulfate and upon the resulting fluid repeat the Phenylhydrazine, Benedict's, Nylander's, and Barfoed's reactions as given for Glucose, pp. 62 ff.

TRISACCHARIDES, $C_{18}H_{32}O_{16}$

RAFFINOSE

This trisaccharide, also called melitose or melitriose, occurs in cotton seed, Australian manna, and in the molasses from the preparation of beet sugar. It is dextrorotatory, does not reduce Benedict's solution, and is only partly fermentable by yeast.

Raffinose may be hydrolyzed by weak acids just as the polysaccharides are hydrolyzed, the products being fructose and melibiose. Further hydrolysis of the melibiose yields glucose and galactose. Raffinose may also be hydrolyzed by the enzyme raffinase, occurring in certain bacteria and yeasts.

POLYSACCHARIDES

The polysaccharides are complex carbohydrates of high molecular weight, either quite insoluble in water or, when soluble, forming colloidal solutions. Polysaccharides in the solid state do not ordinarily appear to be crystalline, but a few crystalline polysaccharides have been isolated and x-ray analysis indicates that certain polysaccharides (e.g., cellulose) possess a definite crystalline structure. Through the action of certain enzymes or of acids the polysaccharides may be hydrolyzed with the formation of simpler compounds which are regarded as constituent units of the polysaccharide. Some polysaccharides yield only simple sugars on hydrolysis; others yield not only sugars but various sugar derivatives such as glucuronic or galacturonic acid (known generally as the *uronic* acids), hexosamines, and even nonsugar compounds such as ethyl alcohol, sulfuric acid, etc.

The constituent units of the polysaccharide molecule appear to be arranged in the form of a long chain, either unbranched (cellulose, amylose) or branched (glycogen, amylopectin). The linkage between units is generally the 1,4 or 1,6 glycoside bond already described, with either the α or β configuration as the case may be. Other types of linkage are known, however. As a class the polysaccharides are nonfermentable and are nonreducing except for a trace of reducing power due presumably to the free reducing group at the end of a chain. They are optically active but do not exhibit mutarotation, and are relatively stable to alkali. This latter fact is utilized for example in the separation of glycogen from tissues prior to analytical determination.

STARCH, $(C_6H_{10}O_5)_x$

Starch is widely distributed throughout the vegetable kingdom, occurring in grains, fruits, and tubers. It is found in cells in the form of granules, the microscopical appearance being typical for each individual starch (see p. 85). The granules differ in size according to the source and they also differ somewhat in composition. The chief constituents are known as amylose and amylopectin, which usually exist in a proportion of about 1:3 in the granule, although some variation in this ratio may be found. Amylopectin appears to contain a small amount of phosphoric acid as a part of the molecule. An amylohemicellulose containing silica has also been reported as present in cereal starches.

When boiled with water, starches form pastes. The starch granules may merely swell without disintegration and thus give a high viscosity to the solutions. Potato-starch granules disintegrate more easily and form less viscous solutions. If starch is ground in a ball mill, much of it disperses readily in water like soluble starch. Soluble starch is formed by the action of dilute hydrochloric acid upon starch. It is so called because it readily forms a limpid, clear solution with hot water. The acid apparently modifies the amylopectin of the granules so that it disintegrates more completely on heating with water.

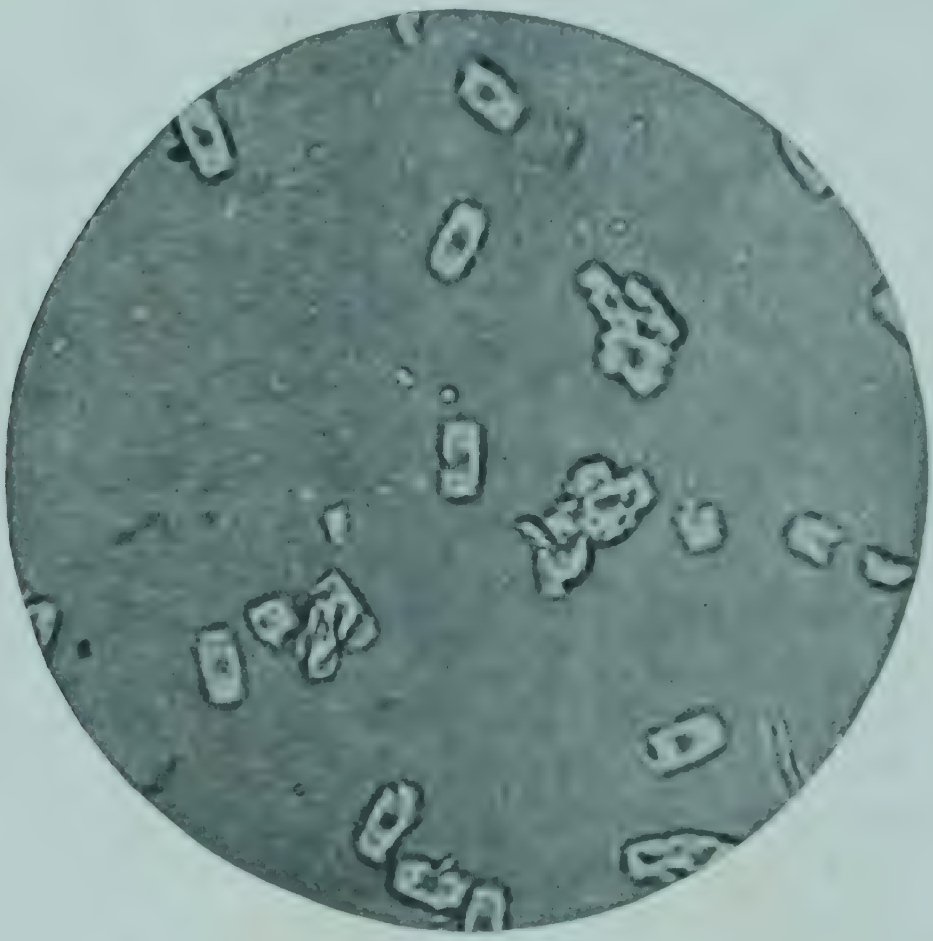
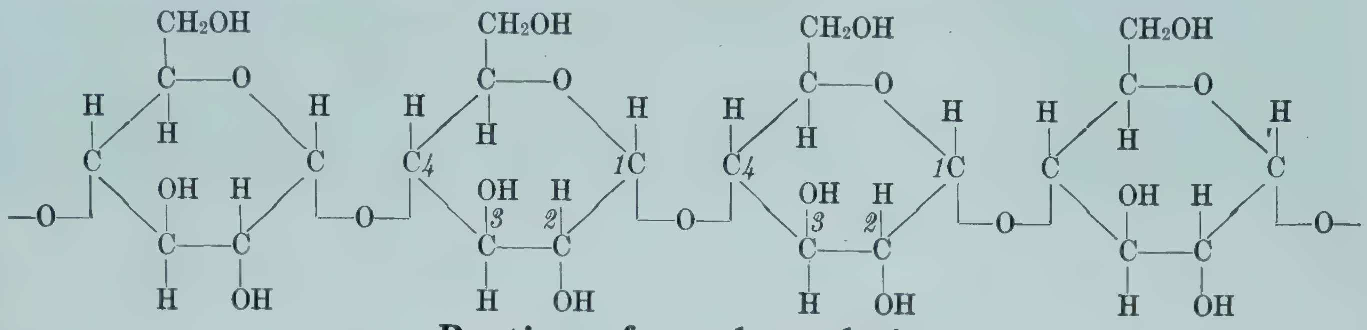


FIG. 20. CRYSTALLINE CORN AMYLOSE.
(Kerr)

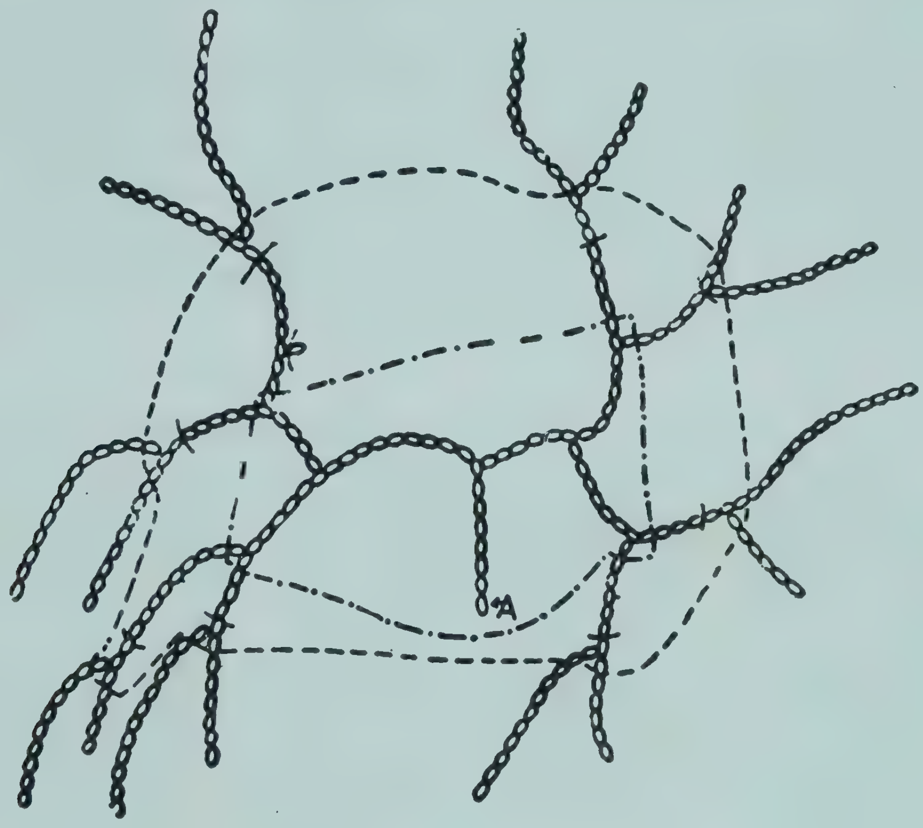
On hydrolysis with acids, starch yields glucose. The two major constituents of the starch granule, amylose and amylopectin, must differ largely, therefore, in their molecular structure. The amylose molecule appears to consist of about 300 glucose units in an unbranched chain, with a molecular weight of about 50,000. When pure, amylose is sparingly soluble in water and forms a clear solution. It gives a deep blue color with iodine, and is completely hydrolyzed to maltose by the enzyme β-amylase. Amylose has been obtained in the crystalline form by Kerr (Fig. 20). Amylopectin on the other hand forms opalescent solutions, gives a purple to violet color with iodine which is much less intense than in the amylose reaction, and is only partly hydrolyzed to maltose by β-amylase. Amylopectin appears to contain many branched glucose chains in the molecule, each chain having about 25 glucose units, and the entire molecule having a molecular weight up to 20 times that of amylose (Fig. 21).

The linkage between glucose units in the unbranched amylose chain is the 1,4 glycoside bond described previously. The structure of a portion of such a chain is probably that indicated in the formula:



Portion of amylose chain

In the branched-chain structure characteristic of amylopectin, both 1,4 and 1,6 glycoside bonds are found; the 1,4 linkage forms the linear portion of a chain and branching takes place by union of a side chain through carbon atom number 1 of a terminal glucose residue of the side chain with carbon atom number 6 of a glucose residue in the main chain.



ooooooooo *Glucose units.*

A *Aldehydic end-group.*

— — — *End of initial degradation by β-amylase, yielding residual-dextrin I.*

(*Limit of degradation produced by α-glucosidase, giving dextrin II, hydrolyzable by β-amylase.*

—·—·— *End of further attack by β-amylase, yielding residual dextrin III.*

FIG. 21. MEYER'S SCHEMATIC REPRESENTATION OF THE BRANCHED AMYLOPECTIN MOLECULE (HASSID).

Courtesy, Wallerstein Communications.

In the course of the digestion of starch by salivary or pancreatic α-amylase there is first formed soluble starch (a clear solution giving a blue color with iodine), then dextrins giving blue or red colors with iodine, next achroodextrins giving no color with iodine, and finally maltose. Some maltose is, however, formed almost from the beginning of the digestion. The amylase apparently catalyzes the hydrolytic splitting of every other glycoside bond, thus producing maltose units. The action of α-amylase on the linear and branched glucose chains, amylose and amylopectin, respectively, is discussed on p. 352. In the case of acid hydrolysis the same intermediate products are formed but glucose is the end product. The hydrolysis of starch by acid is an example of the catalytic action of the hydrogen ion.

Synthetic starches have been prepared by the action of certain muscle and potato enzymes on glucose-1-phosphate, the Cori ester (see p. 333 for an experiment demonstrating such a synthesis). These synthetic starches resemble amylose in their general properties.

EXPERIMENTS ON STARCH

1. *Preparation of Potato Starch.* Pare a raw potato, comminute it upon a fine grater, mix with water, and whip up the pulped material vigorously before straining it through cheesecloth or gauze to remove the coarse particles. The starch rapidly settles to the bottom and can be washed by repeated decantation. Allow the compact mass of starch to drain thoroughly and spread it out on a watch glass to dry in the air. If so desired this preparation may be used in the experiments which follow.

2. *Microscopical Examination.* Examine microscopically the granules of the various starches submitted and compare them with those shown in Figs. 22 to 32. The suspension of the granules in a drop of water will facilitate the microscopical examination.

3. *Solubility.* Try the solubility of one form of starch in cold water, then in hot water. If uncertain regarding the solubility, filter and test the filtrate with iodine solution as given under 5 below. The production of a blue color would indicate that the starch had been dissolved.

4. *Iodine Test.* Place a few granules of starch in one of the depressions of a porcelain test tablet and treat with a drop of a dilute solution of iodine in potassium iodide. The granules are colored blue owing to the formation of a starch-iodine complex.

5. *Iodine Test on Starch Solution.* Place 2 to 3 ml. of dilute starch solution[17] in a test tube, add a drop of the dilute iodine solution, and observe the production of a blue color. Heat the tube and note the disappearance of the color. It reappears on cooling.

In similar tests note the influence of alcohol and of alkali upon the reaction between starch and iodine.

The composition of the blue-colored substance is not well defined. It appears to be an adsorption complex of starch and iodine rather than a definite compound. In performing this test the solution must always be neutral or acid in reaction.

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[17] See Appendix.

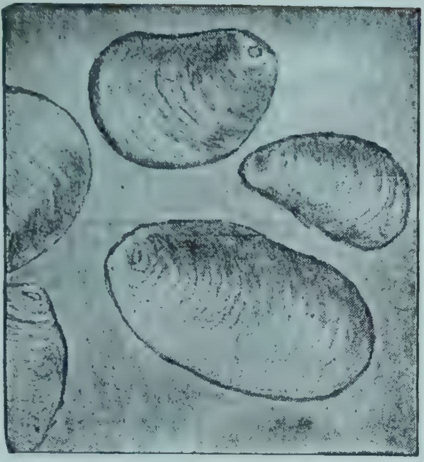


FIG. 22. POTATO.

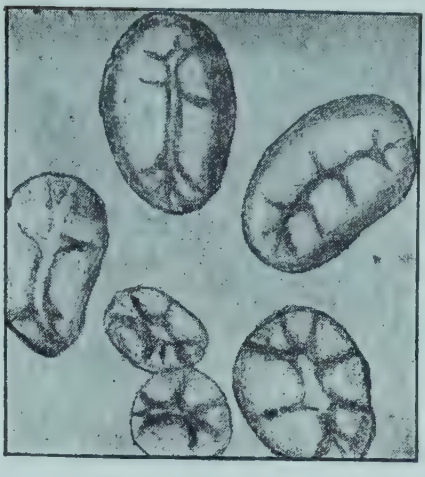


FIG. 23. BEAN.

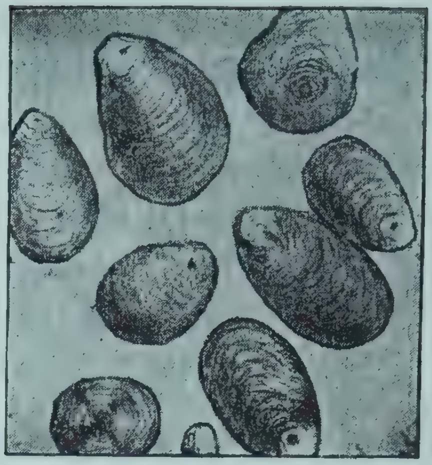


FIG. 24. ARROWROOT.

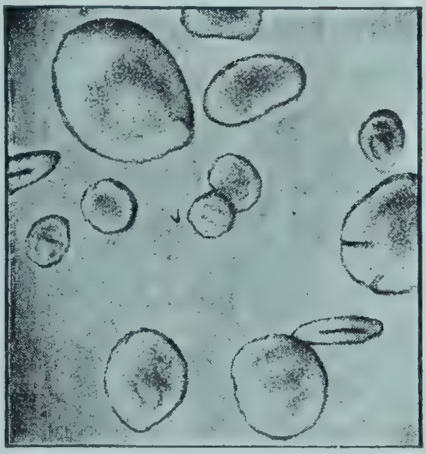


FIG. 25. RYE.

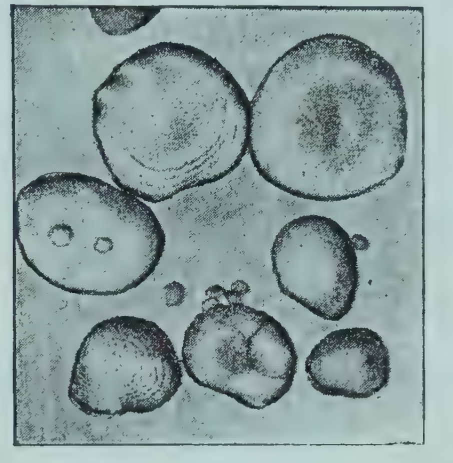


FIG. 26. BARLEY.

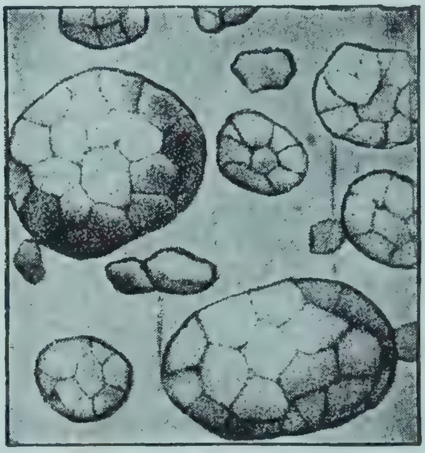


FIG. 27. OAT.

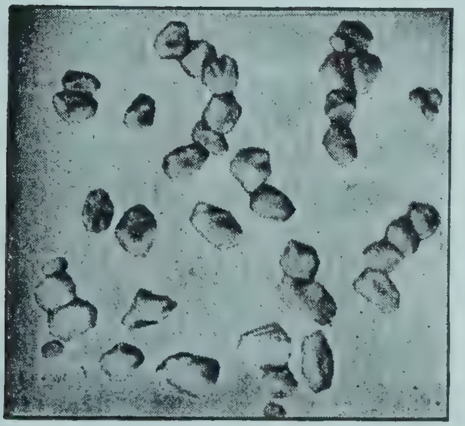


FIG. 28. BUCKWHEAT.

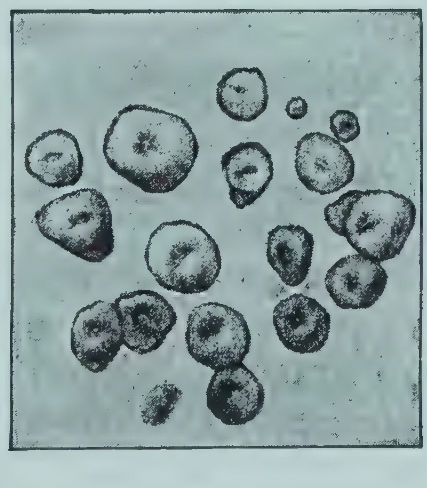


FIG. 29. MAIZE.

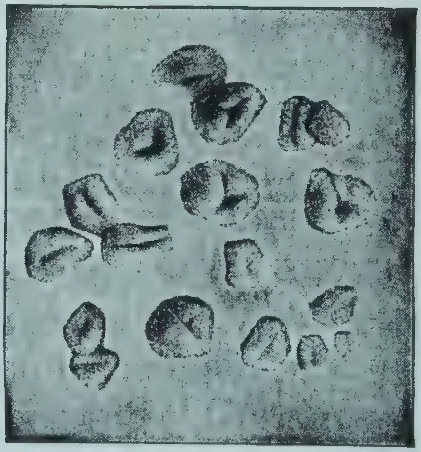


FIG. 30. RICE.

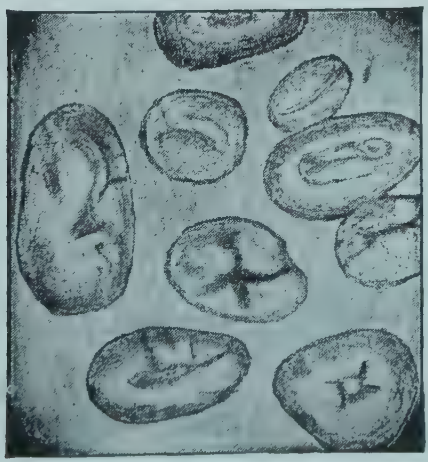


FIG. 31. PEA.

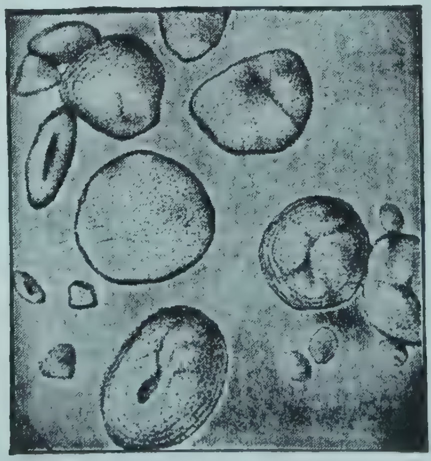


FIG. 32. WHEAT.

FIGS. 22–32. STARCH GRANULES FROM VARIOUS SOURCES.

(Leffmann and Beam)

6. *Benedict's Test.* On starch solution[17] (see p. 66). Does starch have any detectable reducing ability?

7. *Hydrolysis of Starch.* Place about 25 ml. of 1 per cent starch solution[17] in a small beaker, add 10 drops of concentrated HCl, and boil gently. By means of a small pipet, at the end of each minute remove a drop of the solution to the test tablet and make the regular iodine test. At the end of the same 1-minute intervals add exactly 3 drops of the mixture to 5-ml. portions of Benedict's solution in a series of test tubes. As the testing proceeds the reaction with iodine should become weaker and finally be negative. At this point place all the tubes containing Benedict's solution in a boiling water bath for 3 minutes, then remove and allow to cool. Note the degree of reduction in each case and compare with the rate of disappearance of the iodine reaction. Make the phenylhydrazine test upon some of the hydrolyzed starch. What sugar has been formed?

8. *Diffusibility of Starch Paste.* Test the diffusibility of starch through a suitable dialyzing membrane. Compare with glucose in this respect.

INULIN, $(C_6H_{10}O_5)_x$

Inulin is a polysaccharide which may be obtained as a white, odorless, tasteless powder from the tubers of the artichoke, elecampane, or dahlia. It has also been prepared from the roots of chicory, dandelion, and burdock. The rubber-producing plant guayule also contains inulin. It is very slightly soluble in cold water and quite easily soluble in hot water. In cold alcohol of 60 per cent or over it is practically insoluble. Inulin gives a negative reaction with iodine solution. It is very difficult to prepare inulin which does not reduce Benedict's solution slightly. This reducing power may be due to an impurity. Practically all commercial preparations of inulin possess considerable reducing power. Inulin is a polymerized form of fructofuranose, containing about 30 fructofuranose units per chain, united by 1,2 linkages.

Inulin is levorotatory and upon hydrolysis by acids or by the enzyme inulase it yields the monosaccharide fructose which readily reduces Benedict's solution. The preparation of fructose by hydrolysis of the inulin of the Jerusalem artichoke has become of commercial importance. The ordinary amylolytic enzymes occurring in the animal body do not digest inulin. A small part of the ingested inulin may be hydrolyzed by the acid gastric juice, but the value of inulin as a significant source of energy in human dietaries must be questioned. Rats may form some glycogen from inulin. Inulin administered intravenously is readily excreted by the kidneys, apparently because the inulin molecule, though colloidal, is sufficiently small to pass through the renal glomerular membrane. Use is made clinically of this property in the *inulin clearance test* of kidney function and in other studies of renal physiology.

EXPERIMENTS ON INULIN

1. *Solubility.* Try the solubility of inulin powder in hot and cold water and alcohol. If uncertain regarding the solubility, filter and test the filtrate with the Resorcinol-Hydrochloric acid (Selivanoff) test (see below).

2. *Iodine Test.* (a) Place 2 to 3 ml. of the inulin solution in a test tube and add a drop of dilute iodine solution. Compare with a control containing water instead of inulin solution. What do you observe?

(b) Place a small amount of inulin powder in one of the depressions of a test tablet and add a drop of dilute iodine solution. Is the effect any different from that observed above?

3. *Resorcinol-Hydrochloric Acid Test.* Test a portion of inulin solution by this test, following the directions given on p. 73. Explain the results.

4. *Benedict's Test.* Make this test on the inulin solution according to the instructions given for Glucose (p. 66). Is there any reduction? Explain.

5. *Hydrolysis of Inulin.* Place 5 ml. of inulin solution in a test tube, add a drop of concentrated hydrochloric acid, and boil for one minute. Now cool the solution, neutralize it with concentrated sodium carbonate solution, and test the reducing action of 1 ml. of the solution upon 1 ml. of Benedict's solution. Compare with a control using 1 ml. of unhydrolyzed inulin solution. Also try the Resorcinol-Hydrochloric Acid reaction as given on p. 73, likewise comparing with a control of untreated inulin solution. Explain the results.

GLYCOGEN, $(C_6H_{10}O_5)_x$

Glycogen is the form in which carbohydrate is stored in the animal organism. It is found in the liver, muscles, kidneys, and other tissues, but is notably absent from brain. On hydrolysis with amylase it yields maltose, and with acid it yields glucose. It thus resembles starch and the dextrins in these respects but differs significantly from these compounds in molecular architecture. In the glycogen molecule the component glucose residues are linked in chains by both 1,4- and 1,6- α-glycoside bonds as with the starches, to give a highly branched structure as contrasted with the unbranched amylose or slightly branched amylopectin chains of starch (see Fig. 33). Glycogen is soluble in cold water to form an opalescent solution and ordinarily gives a red color with iodine, although some forms of

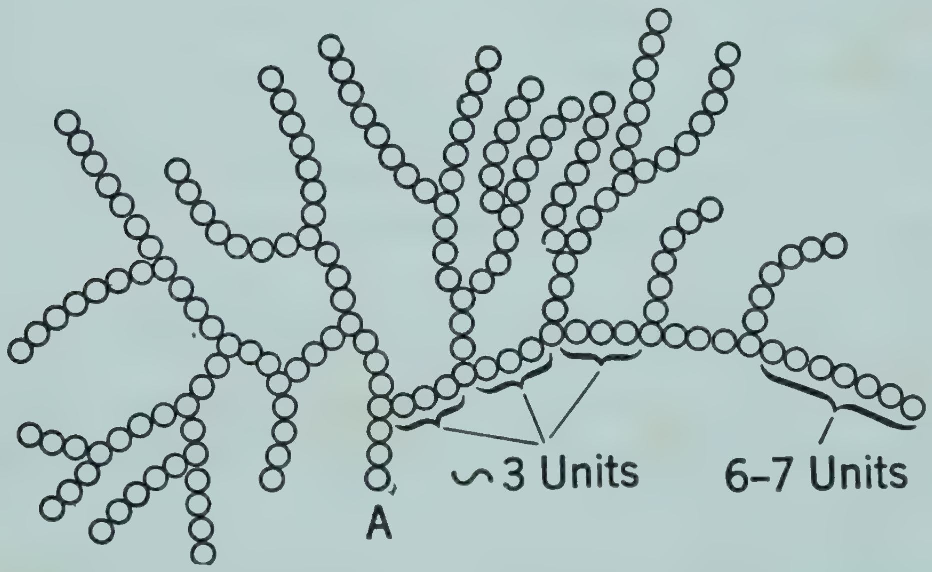


FIG. 33. STRUCTURE OF GLYCOGEN.
The circles represent glucose units; (*A*) the aldehydic end of a chain.
Meyer: *Advances in Enzymology*, 3, 109, 1943.

glycogen which give blue or purple colors with iodine are known. These color differences are apparently related to the extent of chain branching, the blue color representing relatively unbranched chains and the red color with iodine corresponding to highly branched chains.

For a further discussion of glycogen, and experiments, see pp. 272 and 280.

DEXTRIN, $(C_6H_{10}O_5)_x$

The dextrins are found as intermediate products in the course of hydrolysis of starch to glucose or maltose by acids or enzymes. They are colloidal in nature but of a lower degree of molecular complexity than starch. As would be expected, a variety of dextrins are known, most of which are relatively ill-defined, the higher dextrins resembling starch in certain respects while the lower dextrins more nearly resemble the sugars. As a class the dextrins are readily soluble in water, insoluble in alcohol, and do not diffuse through cellulose membranes of ordinary porosity.

The dextrins may be hydrolyzed by dilute acids to form glucose and by amylases to form maltose. They are not fermentable by yeast. Various dextrins give different colors with iodine, depending apparently upon their molecular complexity: the higher dextrins give a blue or purple color, intermediate dextrins give a red color, and the lower dextrins may give no color at all. As is the case with most other polysaccharides, the glucose chains in the dextrin molecule have a free reducing group at one end; the ability of dextrins to reduce Benedict's solution therefore depends upon the molecular weight and chain organization, dextrins with low molecular weight showing detectable reducing power. The reducing ability of commercial dextrin preparations is ordinarily due to the presence of free sugar. The formation of various dextrins as intermediate products of the action of amylases upon starch is illustrated schematically in Fig. 21.

EXPERIMENTS ON DEXTRIN

1. *Solubility.* Test the solubility of pulverized dextrin in hot and cold water. Dextrin forms a clear solution in water, and is thus distinguished from glycogen, which gives an opalescent solution.

2. *Iodine Test.* Place a drop of dextrin solution in one of the depressions of the test tablet and add a dilute solution of iodine in potassium iodide. A red, blue, or purple color results, depending upon the type of dextrin present. Ordinary dextrin preparations may contain some starch, and in the presence of starch it is necessary to have an excess of iodine present. If the reaction is not sufficiently pronounced, make a stronger solution from pulverized dextrin and repeat the test. The solution should be slightly acid to secure the best results.

Make proper tests to show that the red color is influenced by heat, alkali, and alcohol like the blue color given by starch (see p. 84).

The color in the case of dextrin does not reappear as readily on cooling as in the case of starch.

3. *To Detect Dextrin in Presence of Starch.* Treat 5 ml. of dextrin solution with about 10 drops of 1 per cent starch solution. To the mixture add an equal

bulk of saturated ammonium sulfate, shake vigorously, and allow to stand for five minutes. The starch is precipitated. Filter through a dry paper, and to a portion of the filtrate add a drop or two of iodine solution. Compare with an iodine test on the dextrin solution alone which has been treated with ammonium sulfate in like manner.

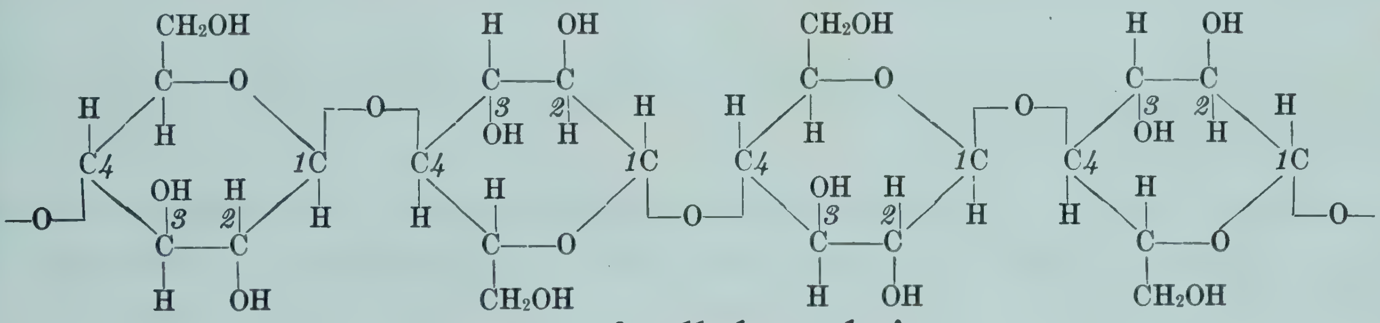
4. *Benedict's Test.* See if the dextrin solution will reduce Benedict's solution.

5. *Precipitation by Alcohol.* To about 50 ml. of 95 per cent alcohol in a small beaker add about 10 ml. of a concentrated dextrin solution. Dextrin is thrown out of solution as a gummy white precipitate.

6. *Diffusibility of Dextrin.* (See Starch, 8, p. 86.)

CELLULOSE, $(C_6H_{10}O_5)_x$

Cellulose forms a large portion of the cell walls of plants. Physically, cellulose is distinguished from other carbohydrates largely by its extreme insolubility in most of the ordinary solvents. Chemically, cellulose has been shown to consist of a large number of β-glucose units joined together in a chain by 1,4-glycoside bonds, as indicated in the formula given herewith. Various methods for estimating the number of glucose units in the cellulose molecule yield results which vary from 200 to 2,000 such units, depending upon the method employed. X-ray studies of cellulose indicate that it is crystalline in structure and consists of bundles of long chains with the chains running parallel to the fiber axis. These chains are unbranched and straight rather than coiled, and there is some evidence for the existence of cross linkages between adjacent chains; the chemical nature of such cross linkages is obscure. The close packing of the long straight chains produces a fiber with much mechanical strength, and may also account for the insolubility of cellulose.



Portion of cellulose chain

Cellulose is not soluble in water or the usual organic solvents nor in dilute acid or alkali. It is soluble however in a variety of special solvents, such as Schweitzer's reagent (ammoniacal copper hydroxide), zinc chloride—hydrochloric acid solution, and sodium hydroxide and CS_2, which latter are the reagents for making viscose, a form of artificial silk. With 10 per cent sodium hydroxide, cellulose is converted into *hydrocellulose* and is said to be mercerized. About 70 per cent sulfuric acid converts it into *vegetable parchment.* Such commercial products as rayon, cellophane, sausage casing, etc., are made from cellulose. With strong nitric acid and concentrated sulfuric acid it forms nitrocellulose.

Cellulose is not hydrolyzed by boiling with dilute mineral acids. It may be hydrolyzed, however, by treating with concentrated sulfuric acid, then diluting the solution with water, and boiling. The product of this hydrolysis is glucose. Hydrolysis of cellulose by certain bacteria has been reported to yield the disaccharide cellobiose, analogous to the production of maltose from starch.

There is some difference of opinion as to the exact extent to which cellulose is utilized in the animal organism. It is no doubt more efficiently utilized by herbivora than by carnivora or by man. It is claimed that about 25 per cent may be utilized by herbivora and less than 5 per cent by dogs, whereas the quantity utilized by man is too small for it to play a nutritional role in the diet of a normal individual. In neither man nor the lower animals has there been demonstrated any formation of sugar or glycogen from cellulose. It is probable that the cellulose which disappears from the intestine is transformed for the most part into lower fatty acids (acetic, etc.) by the action of intestinal bacteria. A *cellulase* capable of digesting sawdust and filter paper has been found in the digestive diverticula attached to the stomach of the shipworm.

EXPERIMENTS ON CELLULOSE

For these experiments, a high-grade filter paper, absorbent cotton, or cleansing tissue may be used.

1. *Solubility.* Test the solubility of cellulose in water, dilute and concentrated acid, and alkali.

2. *Iodine Test.* Add a drop of dilute iodine solution to a few shreds of cotton on a test tablet. Cellulose differs from starch and dextrin in giving no color with iodine.

3. *Formation of Amyloid.*[18] To 6 ml. of distilled water in a test tube, add 10 ml. of concentrated sulfuric acid. The acid should be added to the water in small portions and the mixture stirred with a stirring rod and cooled under the tap, or by immersion in a beaker of cold water, between additions. To the cooled mixture add a two-inch square of cleansing tissue and stir for from 5 to 10 minutes, when most of the tissue is dissolved. Pour about 3 ml. of this solution into about 10 ml. of distilled water, and note the flocculent precipitate of amyloid formed. To another small portion of the solution add iodine and note the blue or black color formed. Pour the remainder of the acid solution of tissue into about 25 ml. of distilled water in a small beaker and boil for 15 to 30 minutes. Now cool, neutralize with solid sodium carbonate, and test with Benedict's solution. Glucose has been formed from the cellulose by the action of the acid.

4. *Ammoniacal Cupric Hydroxide Solubility Test (Schweitzer).* Place a 2-inch square of cleansing tissue in a test tube, add 5 ml. of Schweitzer's reagent,[19] and stir the cellulose with a glass rod. When completely dissolved (5 to 10 minutes), dilute with an equal volume of distilled water, and acidify the solution with acetic acid. An amorphous precipitate of cellulose is produced.

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[18] This substance derives its name from *amylum* (starch) and is not to be confounded with amyloid, the glycoprotein.

[19] See Appendix.

5. ***Hydrochloric Acid—Zinc Chloride Solubility Test*** **(*Cross and Bevan*). Place a little absorbent cotton in a test tube, add Cross and Bevan's reagent,[19] and stir the cellulose with a glass rod. When solution is complete, reprecipitate the cellulose with 95 per cent alcohol.**

6. ***Iodine—Zinc Chloride Reaction.*** **Place a little absorbent cotton or quantitative filter paper in a test tube and treat it with the iodine—zinc chloride reagent.[19] A blue color forms on standing. Amyloid has been formed from the cellulose through the action of the $ZnCl_2$ and the iodine solution has stained the amyloid blue.**

7. ***Other Cellulose Solvents.*** **It has been demonstrated by Deming that there are many excellent solvents for cellulose (filter paper)—for example, the concentrated aqueous solutions of certain salts such as (1) antimony trichloride, (2) stannous chloride, and (3) zinc bromide. In hydrochloric acid solution the solvent action of the above salts is increased. The following salts are also good solvents in hydrochloric acid solution: mercuric chloride, bismuth chloride, antimony pentachloride, tin tetrachloride, and titanium tetrachloride. In the case of the last-mentioned salt the swollen, transparent character of the cellulose fibers preliminary to solution can be seen very nicely.**

Try selected solvents suggested by the instructor.

HEMICELLULOSES

The hemicelluloses differ from cellulose in that they may be hydrolyzed upon boiling with dilute mineral acids. They differ from other polysaccharides in being not readily digested by amylases. Upon hydrolysis hemicellulose may yield pentoses or hexoses or both, together with uronic acids. The vegetable gums and pectins may be included under this head.

Pentosans. Pentosans yield pentoses upon hydrolysis. So far as is known they do not occur in the animal kingdom. They have, however, a very wide distribution in the vegetable kingdom, being present in leaves, roots, seeds, and stems of all forms of plants, many times in intimate association or even chemical combination with galactans and uronic acids. In herbivora, pentosans are 40 to 80 per cent utilized. The few tests on record as to the pentosan utilization by man indicate that 80 to 95 per cent disappears from the intestine. According to Cramer, bacteria are efficient hemicellulose transformers. It has not yet been demonstrated that pentosans form glycogen in man, and for this reason they must be considered to play an unimportant part in human nutrition. Gum arabic, an important pentosan, may be hydrolyzed by boiling with strong hydrochloric acid for a short time. The pentose arabinose results from such hydrolysis.

Galactans. In common with the pentosans the galactans have a very wide distribution in the vegetable kingdom. One of the most important members of the galactan group is agar-agar, a product prepared from certain types of Asiatic or American seaweed. Chemically, the agar molecule appears to consist of a chain of D-galactose units linked by a 1,3-glycoside bond, with a single L-galactose unit in a 1,4 linkage at the reducing end of the chain, this latter unit also being esterified in the 6 position with sulfuric acid. Thus the products of hydrolysis include much D-galactose, some L-galactose, and sulfuric acid. This galactan is about 50 per cent utilizable by herbivora and 8 to 27 per cent utilizable by man. Agar ingestion has been shown to be a very efficient therapeutic aid in cases of chronic constipation. This is particularly true when the constipation is due to the formation of dry, hard fecal masses (scybala), a type of fecal formation which frequently follows the ingestion of a diet which is very thoroughly digested and absorbed. The agar, because of its relative indigestibility and its property of absorbing water, yields a bulky fecal mass which is sufficiently soft to permit of easy evacuation. Agar has been used with good results in the treatment of constipation in children. Agar is not limited to its use in connection with constipation, but may serve in other capacities as an aid to intestinal therapeutics.

Dextrans and Levans. The dextrans are polysaccharides produced by the action of certain bacteria on sucrose, the reaction being as follows:

$$\underset{\textbf{Sucrose}}{nC_{12}H_{22}O_{11}} = \underset{\textbf{Fructose}}{nC_6H_{12}O_6} + \underset{\textbf{Dextran}}{(C_6H_{10}O_5)_n}$$

In effect, the fructose residue is removed from the sucrose molecule, and the remaining glucose residues polymerized to form the polysaccharide dextran. The dextrans have a high molecular weight (which varies with the conditions of preparation and species of organism used) and form rather viscous colloidal solutions in water. For many years they were regarded as undesirable by-products in the sugar and fermentation industries. They have attracted considerable clinical interest because of their proposed used as plasma substitutes, since they form nontoxic solutions with a high colloidal osmotic pressure, suitable for intravenous administration as a temporary replacement for plasma proteins lost from the blood by hemorrhage. Dextrans so administered gradually disappear from the circulation; their metabolic fate is however still obscure.

The dextrans consist almost entirely of D-glucose units joined in chains by 1,6-glycoside bonds, with evidence for occasional cross-linking between chains by the formation of 1,4 bonds. On hydrolysis with dilute acids, dextrans yield only D-glucose. In general, dextrans are not acted upon by ordinary amylases, although some exceptions to this rule are known.

Similar polysaccharides containing fructose residues and known as levans are also produced by certain microorganisms. The reaction is analogous to that given for dextran formation, with the formation of glucose and polymerized fructose (levan), except that the incidence of side reactions involving the breakdown of part of the sucrose to free glucose and fructose appears to be greater. Chemically, levans are found to be polymers of fructose with 2,6 linkages between units, thus differing from inulin, which contains 1,2 linkages. Levan formation has relatively little commercial interest at the present time.

The Pectins. The pectins are colloidal carbohydrates which with the proper concentration of acid and of sugar form gels. For a gel to form, there must be present from 0.3 to 0.7 per cent pectin, 65 to 70 per cent of sugar (usually sucrose), and a pH of 3.2 to 3.5. Commercial pectin is prepared from apples and lemons. On hydrolysis the pectins yield galacturonic acid, arabinose, galactose, acetic acid, and methyl alcohol. The characteristic properties of pectin appear related to the presence of a long chain of anhydrogalacturonide residues, partly methyl esterified. Nongalacturonide material, galactan, and araban, which appear to act chiefly as diluents, may however make up a considerable portion of the weight of the pectin.

EXPERIMENTS ON A PENTOSAN

1. *Solubility*. Test the solubility of gum arabic in hot and cold water and alcohol.

2. *Iodine Test*. Add a drop of dilute iodine solution to a little gum arabic on a test tablet. It resembles cellulose in giving no color with iodine.

3. *Hydrolysis of Gum Arabic*. Introduce a little gum arabic into a test tube, add 5 to 10 ml. of strong hydrochloric acid (conc. HCl and water 1:1), and heat to boiling for 5 to 10 minutes. Cool, neutralize with sodium hydroxide, and test by the Benedict or some other reduction test. A positive reaction should be obtained, indicating that the gum arabic has been hydrolyzed by the acid with the production of a reducing substance. What is this reducing substance? How would you identify it?

EXPERIMENTS ON A GALACTAN

1. *Solubility*. Test the solubility of agar-agar in hot and cold water. Observe its marked property of imbibing water (see above).

2. *Iodine Test*. Add a drop of dilute iodine solution to a little agar-agar on a test tablet. It resembles cellulose in giving no color with iodine.

3. *Hydrolysis of Agar-agar*. Introduce a few pieces of agar-agar in a test tube, add 5 to 10 ml. of strong hydrochloric acid (conc. HCl and water 1:1) and heat to boiling for 5 to 10 minutes. Cool, neutralize with sodium hydroxide, and test by the Benedict or some other reduction test. A positive reaction should be obtained, indicating that the agar-agar has been hydrolyzed by the acid with the production of a reducing substance. What is this reducing substance? How would you identify it?

EXPERIMENTS ON PECTIN

1. *Preparation of Pectin*. Pare off the yellow layer from a grapefruit rind. Run through a meat chopper, cover with water, and let stand over night. Strain on cheesecloth and squeeze out the fluid. Boil the pulp with water for about two hours, bringing finally to a low volume. Pour off the fluid and add alcohol to precipitate the pectin. Filter and dry.

2. *Formation of a Gel*. Into a weighed 400-ml. beaker introduce 70 g. of cane sugar, 1 g. of dry pectin, 0.5 g. of citric or tartaric acid, and 100 ml. of water. Heat to boiling. Concentrate to a weight of 100 g. Let it stand over night.

REVIEW OF CARBOHYDRATES

In order to facilitate the student's review of the carbohydrates, the preparation of a chart similar to the appended model is recommended. The signs + and − may be conveniently used to indicate positive and negative reaction. Only those carbohydrates which are of greatest importance from the standpoint of physiological chemistry have been included in the following model chart.

Carbohydrate	*Solubility*	*α-Naphthol Reaction (Molisch)*	*Benedict's Test*	*Nylander Test*	*Barfoed's Test*	*Iodine Test*	*Resorcinol—Hydrochloric Acid Reaction (Selivanoff)*	*Tauber's Benzidine Test*	*Mucic Acid Test*	*Precipitation by Alcohol*	*Osazone*	*Rotation*	*Diffusibility*	*Fermentation*	*Products of Hydrolysis*	*Remarks*
Glucose																
Fructose																
Galactose																
Pentose																
Maltose																
Lactose																
Sucrose																
Starch																
Inulin																
Glycogen																
Dextrin																
Cellulose																
Gum arabic																
Agar-agar																

QUANTITATIVE DETERMINATION OF CARBOHYDRATES

See the chapters on Blood, Milk, and Urine for methods applicable also to pure solutions.

SCHEME FOR THE DETECTION OF CARBOHYDRATES

If the solution is alkaline, neutralize or make faintly acid with HCl. Test a few drops on a test tablet with iodine solution.

- Blue color indicates **Starch** and possible **Dextrins.***
- Reddish color indicates
  - **Dextrin** if unknown solution is clear.
  - **Glycogen** if unknown solution is opalescent.
  - If uncertain as to the differentiation between dextrin and glycogen, add ammoniacal basic lead acetate to some of the unknown solution.
    - No precipitate indicates **dextrin.**
    - Precipitate indicates **glycogen.**
- No color with iodine solution indicates possibility of **sugars.**†
  - Test the unknown solution by Benedict's test.
    - Reduction indicates **glucose, fructose, mannose, galactose, pentose, maltose, lactose,** possibly along with nonreducing carbohydrates.
      - Test the unknown with Barfoed's solution.
        - Reduction indicates **glucose, fructose, mannose, galactose, pentose,** possibly along with nonreacting carbohydrates.
          - **Test** the unknown by **resorcinol**—hydrochloric acid (Selivanoff). Positive test indicates **fructose** if sucrose and inulin are absent. Sucrose if present will already have been detected. Absence of inulin is shown by lack of turbidity on treating the unknown with 3 volumes of 95 per cent alcohol.
          - Treat the unknown with phenylhydrazine reagent as for osazone test. Formation of colorless insoluble phenylhydrazone before heating indicates **mannose.** Heat to complete osazone reaction. Formation of yellow insoluble osazone during heating indicates **glucose, fructose, mannose.** Osazone separating out only after cooling indicates **lactose, maltose.** Examine crystals under microscope to identify various sugars.
          - Try mucic acid test on the unknown. Positive test indicates **lactose, galactose.** Differentiate by Barfoed test, osazone.
          - Try benzidine reaction (Tauber) on the unknown. Positive test indicates **pentose** or **pentosan.** Absence of pentosan is shown by lack of turbidity on treatment of unknown with 3 volumes of 95 per cent alcohol.
          - Rub up a portion of unknown solution with yeast, and allow to stand until fermentation is complete. Filter and test filtrate by Benedict test. Reduction before and after yeast treatment indicates **galactose, lactose, pentose;** reduction before but not after fermentation indicates **glucose, fructose, mannose, maltose.**
        - No reduction indicates **lactose,** or **maltose** or both. Differentiate by osazone reaction, mucic acid test, or fermentation test.
      - If Benedict's solution is reduced, boil with excess reagent until filtrate no longer gives a reduction on further boiling. Filter, acidify filtrate with conc. HCl, boil for one minute, cool, neutralize with conc. Na_2CO_3, and test again with Benedict's solution. Reduction indicates the presence of **sucrose** in the unknown solution.
    - No reduction indicates the possible presence of **sucrose.**
      - To 5 ml. of the unknown solution in a test tube, add one drop of concentrated HCl and boil for one minute. Cool, neutralize with concentrated Na_2CO_3 solution, and test by Benedict's test.
        - Reduction indicates the presence of **sucrose** in the unknown solution.
        - No reduction indicates the absence of sucrose in the unknown solution.

---

* **Starches** may be precipitated from solution by addition of an equal volume of saturated ammonium sulfate solution.

† If iodine test indicates the presence of **polysaccharides** in the unknown solution, these may be precipitated from solution, before testing for the simple sugars, by the addition of 3 volumes of 95 per cent alcohol. Excess alcohol may be evaporated from the neutral filtrate.

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3

Fats

THE LIPIDES

The term lipide is applied to a group of naturally occurring substances characterized by their insolubility in water and their solubility in such *fat solvents* as ether, chloroform, boiling alcohol, and benzene. They are limited to substances which are utilizable by the animal organism. Individual members of this group show large individual variations in solubility, but as a class the lipides are readily distinguishable from the carbohydrates and the proteins, the other two great groups of naturally occurring compounds. Chemically, the lipides are either esters of fatty acids or substances capable of forming such esters. They are very widespread in nature, being found in all vegetable and animal matter. Some members of this group, such as the phosphatides and sterols, are found in all living cells where, with the proteins and carbohydrates, they form an essential part of the colloidal complex of protoplasm. Complex lipides are also found in large quantities in brain and nervous tissues, thus indicating the important role these substances must play in the living organism. Other lipides, such as the fats and oils, represent the chief form in which excess nutrients are stored in the animal body. They arise from ingested lipides and from the metabolism of carbohydrates and proteins, and are stored in fat deposits, such as in the subcutaneous connective tissue, in the intermuscular connective tissue, in the omentum, in the perirenal fat depots, and in the genital fat. Lipides act as heat insulators and as reserve supplies of energy. They may be classified as follows:

CLASSIFICATION

SIMPLE LIPIDES

The simple lipides are esters of fatty acids with certain alcohols. They are usually further classified according to the nature of the alcohols, as follows:

1. Fats and Oils. Esters of fatty acids and glycerol. Oils are fats which are liquid at room temperature.

2. Waxes. Esters of fatty acids with long-chain aliphatic alcohols or with cyclic alcohols. These may be subdivided into: (1) True waxes; (2) Cholesterol esters; (3) Vitamin A and its carotenol esters; and (4) Vitamin D esters.

COMPOUND OR CONJUGATE LIPIDES

The compound lipides are esters of fatty acids which, on hydrolysis, yield other substances in addition to fatty acids and an alcohol. Some important members of this group are:

1. Phospholipides (Phosphatides). Lipides which, on hydrolysis, yield fatty acids; phosphoric acid; sometimes, but not always, glycerol; and a nitrogenous base. These are subdivided into the following groups:

(a) LECITHINS. Lipides containing fatty acids, phosphoric acid, glycerol, and the nitrogenous base choline.

(b) CEPHALINS. Lipides which yield on hydrolysis fatty acids, glycerol, phosphoric acid, and either the base ethanolamine (colamine) or the amino acid serine. Lipides of uncertain structure which contain inositol, fatty acids, phosphoric acid, ethanolamine, and possibly galactose and tartaric acid are also included in this class.

(c) SPHINGOMYELINS. Lipides containing the nitrogenous base sphingosine, a single fatty-acid molecule, phosphoric acid, and choline, but no glycerol.

2. Cerebrosides. Lipides which contain carbohydrate (galactose or glucose), one fatty acid, and sphingosine, but no phosphoric acid or glycerol.

3. Sulfolipides. Similar to cerebrosides except that sulfuric acid is present as cerebronic acid ester. Sulfolipides containing sphingosine, galactose, cerebronic acid, sulfuric acid, and potassium have been described.[1]

DERIVED LIPIDES

The derived lipides are substances formed in the hydrolysis of simple or compound lipides which still retain the properties of this class of compound.

1. Fatty Acids. Saturated and unsaturated acids.

2. Alcohols. Compounds of high molecular weight but not gycerol. These may be classified as follows:

(a) ALIPHATIC ALCOHOLS such as cetyl ($CH_3(CH_2)_{14}CH_2OH$), stearyl ($CH_3(CH_2)_{16}CH_2OH$), and myricyl ($CH_3(CH_2)_{28}CH_2OH$).

(b) STEROLS which contain the phenanthrene nucleus (cholesterol, ergosterol, sitosterol, stigmasterol).

(c) ALCOHOLS CONTAINING THE β-IONONE RING. These include vitamin A, kitol, and carotenols such as cryptoxanthin, lutein, and zeaxanthin.

3. Hydrocarbons. Compounds having no carboxyl or alcohol groups, and which cannot be saponified.

(a) ALIPHATIC HYDROCARBONS. Pentacosane, $CH_3(CH_2)_{23}CH_3$, and homologues to hentriacontane, $C_{31}H_{64}$.

(b) CAROTENOIDS. $C_{40}H_{56}$ compounds, such as α-, β-, and γ-carotene and lycopene.

(c) SQUALENE. Unsaturated hydrocarbon, $C_{30}H_{56}$, in olive and shark-liver oils.

4. Vitamins D. Differ from steroids in that the phenanthrene nucleus is ruptured between carbons 9 and 10.

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[1] Blix: *Z. physiol. Chem.*, **219**, 82 (1933).

5. Vitamins E. Chroman derivatives, α-, β-, γ-, and δ-tocopherols.

6. Vitamins K. Derivatives of 1,4-naphthoquinone with long hydrocarbon side chains.

THE FATS

The Fatty Acids. The fatty acids found in fats and other lipides are of various types. Some of them, like palmitic acid ($CH_3(CH_2)_{14}COOH$) and stearic acid ($CH_3(CH_2)_{16}COOH$), are straight-chain saturated acids belonging to the acetic acid series, and have the general formula $C_nH_{2n}O_2$. Others are unsaturated and have from one to four, and even more, double bonds in their molecules. Thus oleic acid ($C_{18}H_{34}O_2$) has one double bond in its molecule, linoleic acid ($C_{18}H_{32}O_2$) has two double bonds, and linolenic acid ($C_{18}H_{30}O_2$) has three. Practically all the fatty acids found in nature contain an even number of carbon atoms, although porpoise oil and the head oil of the white whale have been shown to contain isovaleric acid ($C_5H_{10}O_2$). In addition to the above types of acid, some lipides contain hydroxy acids, both saturated and unsaturated, and dicarboxylic acids. Thus castor oil contains an unsaturated hydroxy acid, ricinoleic acid ($C_{18}H_{34}O_3$). Certain cyclic saturated and unsaturated fatty acids are likewise found in nature. Some of the more important chemical and physical properties of saturated and unsaturated fatty acids are discussed below.

SATURATED FATTY ACIDS, $C_nH_{2n}O_2$ OR $C_nH_{2n+1}COOH$

The physical properties of the saturated fatty acids depend upon their molecular weights. Whereas those fatty acids that contain ten carbon atoms or fewer in their molecules are liquids at room temperature, the remainder are solids whose melting points rise with increasing molecular weight. The liquid acids are also known as volatile fatty acids, since they may be distilled with steam, whereas the others, the nonvolatile acids, are

SATURATED FATTY ACIDS

Common Name	*Chemical Name*	*Structure*	*Occurrence*
Butyric.......	Butanoic	$CH_3\cdot(CH_2)_2\cdot COOH$	Butter fat
Caproic.......	Hexanoic	$CH_3\cdot(CH_2)_4\cdot COOH$	Butter fat, coconut oil
Caprylic......	Octanoic	$CH_3\cdot(CH_2)_6\cdot COOH$	Butter fat, coconut oil
Capric........	Decanoic	$CH_3\cdot(CH_2)_8\cdot COOH$	Butter fat, coconut oil
Lauric........	Dodecanoic	$CH_3\cdot(CH_2)_{10}\cdot COOH$	Laurel kernel oil, butter fat, coconut oil
Myristic......	Tetradecanoic	$CH_3\cdot(CH_2)_{12}\cdot COOH$	Nutmeg fat, butter fat, vegetable fats
Palmitic......	Hexadecanoic	$CH_3\cdot(CH_2)_{14}\cdot COOH$	Most vegetable and animal fats
Stearic.......	Octadecanoic	$CH_3\cdot(CH_2)_{16}\cdot COOH$	Most vegetable and animal fats
Arachidic.....	Eicosanoic	$CH_3\cdot(CH_2)_{18}\cdot COOH$	Peanut oil
Behenic......	Docosanoic	$CH_3\cdot(CH_2)_{20}\cdot COOH$	Rapeseed oil, peanut oil
Lignoceric....	Tetracosanoic	$CH_3\cdot(CH_2)_{22}\cdot COOH$	Cerebrosides, sphingomyelin, peanut oil

carried over by steam distillation only in traces or not at all. Fatty acids with four carbon atoms or fewer are miscible with water in all proportions. As the length of the carbon chain increases beyond this, however, the solubility rapidly diminishes to zero. The common straight-chain saturated fatty acids found in nature as constituents of lipide molecules are listed in the table on p. 99.

UNSATURATED FATTY ACIDS

The unsaturated fatty acids are characterized by the presence of one or more double bonds in the molecule. They have been classified in accordance with the number of double bonds as monoethenoid, diethenoid, triethenoid, etc., and named by reference to the parent hydrocarbon, the position of the double bond or bonds in the chain being indicated by a number referred to the carboxyl carbon atom as number one. As is true of the saturated fatty acids, many of the unsaturated fatty acids have common names which may be used as frequently as the chemical name.

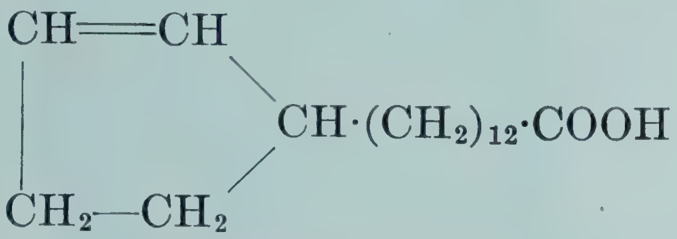
Because of the presence of the double bond the unsaturated fatty acids are much more reactive than the saturated acids, the reactivity increasing with increase in the number of double bonds. The unsaturated fatty acids are capable of taking up one molecule of water, oxygen, hydrogen, bromine, or iodine at each double bond, and the amount of such substance (e.g., iodine) absorbed by a given weight of acid is used to determine its degree of unsaturation. It is obvious that a variety of isomerism is possible among the unsaturated fatty acids, depending not only on the position of the double bond in the chain but also on *cis-trans* isomerism across a double bond. Relatively few of the large number of possible isomers of the unsaturated fatty acids are found in nature.

The most common unsaturated fatty acid found in nature is the monoethenoid acid oleic acid (9-octadecenoic acid). This acid is so widely distributed that, according to Hilditch, no natural fat or phosphatide has as yet been found not to contain oleic acid. Vaccenic acid, an isomer of oleic acid, has received considerable attention. It occurs in low concentration in certain animal and vegetable tissues. Other common unsaturated fatty acids include (1) the diethenoid acid linoleic acid, found in both animal and plant fats; (2) linolenic acid, which is a triethenoid acid found largely in vegetable fats, but also in some animal fats (horse, egg yolk); and (3) arachidonic acid, a tetraethenoid acid which is found in both the fat and the phosphatide fractions of many animal tissues, particularly in liver and in suprarenal phospholipides. The chemical characteristics of these representative straight-chain unsaturated fatty acids follow:

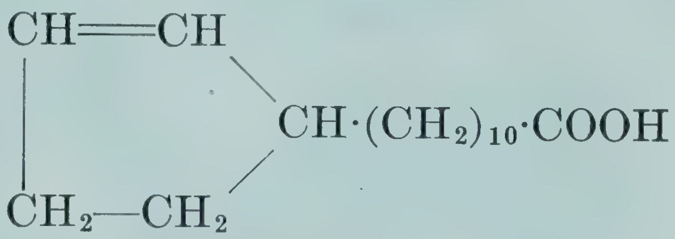
Common Name	*Empirical Formula*	*Number of Double Bonds*	*Chemical Name*
Oleic Acid	$C_{18}H_{34}O_2$	1	9-Octadecenoic
Linoleic Acid	$C_{18}H_{32}O_2$	2	9,12-Octadecadienoic
Linolenic Acid	$C_{18}H_{30}O_2$	3	9,12,15-Octadecatrienoic
Arachidonic Acid	$C_{20}H_{32}O_2$	4	5,8,11,14-Eicosatetraenoic

From the empirical formulas it can be seen that oleic, linoleic, and linolenic acids may be considered derivatives of the saturated C_{18} acid, stearic acid, and arachidonic acid is an unsaturated form of the C_{20} acid, arachidic acid. Similar ethenoid derivatives are known of many of the other saturated fatty acids found in nature.

In addition to the straight-chain fatty acids so far described, a number of branched-chain and cyclic fatty acids, both saturated and unsaturated, have been isolated from natural sources. Tuberculostearic acid (10-methylstearic acid) has been obtained from the wax of the tubercle bacillus, as has phthioic acid, a methylated branched chain C_{26} acid which is possibly 3,13,19-trimethyltricosanoic acid, although this structure has been disputed. The latter appears to be associated with some of the clinical manifestations of tuberculosis. It is interesting to note that these and other fatty acids are found in tubercle wax as esters of the disaccharide trehalose (see Chapter 2). In chaulmoogra oil the unsaturated cyclic fatty acids chaulmoogric acid and hydnocarpic acid are found; these acids or their derivatives have been used in the treatment of leprosy.

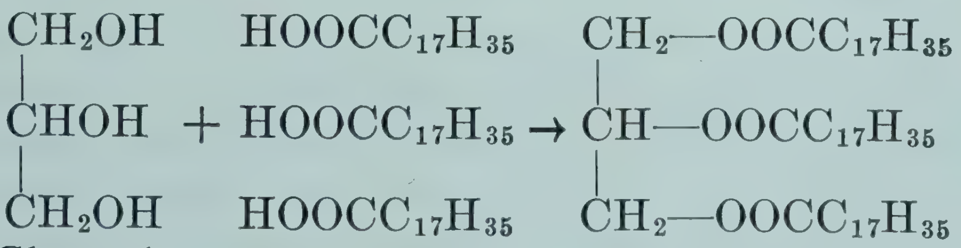


Chaulmoogric acid



Hydnocarpic acid

The Fats. The fats are neutral esters of glycerol and fatty acids. An example is tristearin, synthesized in living tissues from one molecule of glycerol and three molecules of stearic acid:



$$\begin{array}{lcl} \begin{array}{l} CH_2OH \\ | \\ CHOH \\ | \\ CH_2OH \end{array} & + \begin{array}{l} HOOCC_{17}H_{35} \\ \\ HOOCC_{17}H_{35} \\ \\ HOOCC_{17}H_{35} \end{array} \rightarrow & \begin{array}{l} CH_2—OOCC_{17}H_{35} \\ | \\ CH—OOCC_{17}H_{35} \\ | \\ CH_2—OOCC_{17}H_{35} \end{array} \end{array}$$

Glycerol **Stearic acid** **Tristearin**

Of the three molecules of fatty acid entering into the composition of a "true" or neutral fat, all may be the same, as in tristearin, in which case it is referred to as a simple triglyceride. However, more commonly two or three different fatty acids are involved, and such a fat is called a mixed glyceride, the composition being indicated by the name as oleodipalmitin, stearodiolein, oleopalmitostearin, etc. Mixed glycerides occur much more commonly than do simple glycerides such as tristearin, tripalmitin, and triolein. The possibilities of stereoisomerism among the fats are many; they include isomerism due to the same fatty acids being arranged differently in the molecule, as well as optical isomerism when the middle carbon atom of the glycerol portion becomes asymmetric or when an optically active fatty acid is present. Such isomerism appears to be of much less physiological importance in the fats than in carbohydrates and proteins. Diglycerides (containing two fatty acids) and monoglycerides (containing one fatty acid) have been shown to be normal intermediates arising in fat digestion. The 1,2-diglyceride and the 2-monoglyceride are believed to be the primary types.[2]

Animal Fats. Naturally occurring animal fats consist largely of mixed glycerides of oleic, palmitic, and stearic acid; furthermore they are usually mixtures of individual fats. Fats from various sources differ considerably in their fatty acid composition. Mutton fat contains more stearic acid and less oleic acid than pork fat. Human fats contain a high percentage of oleic acid. Butter fat consists largely of glycerides of palmitic and oleic acids, with small amounts of stearic acid and of the lower fatty acids such as butyric and caproic; significant variation in this connection may be noted from species to species (see Chapter 12).

General Properties of Fats. Pure neutral fats are odorless, tasteless, and generally colorless; the color of natural fats and oils is ordinarily due to pigments mixed with or dissolved in the fat. In the so-called *colored fats*, however, the chromogenic material is present in combination. Examples are chlorophyll, in which a colored acid is combined with a colorless alcohol (phytol); and colored waxes, in which colorless acids are esterified with colored alcohols (xanthophyll, capsanthin).[3] Fats are insoluble in all aqueous solvents, but are readily soluble in ether, benzene, chloroform, *boiling* alcohol, and other organic solvents, and this is the common basis for separating fats from other substances. The neutral fats are nonvolatile, and may be crystallized, some of them with great facility. The crystalline forms of some of the more common fats are reproduced in Figs. 34, 35, and 36. Each individual fat possesses a specific melting point, which depends upon the nature of the fatty acids in the molecule, and this property of melting at a definite temperature may be used as a means of differentiation if the fat is sufficiently pure. Tristearin, for example, melts at 71° C., tripalmitin at 66° C., and triolein at $-5°$ C. In the glycerides containing saturated fatty acids, increase in molecular weight of the component fatty acids results in a higher melting point; the change from a saturated to an unsaturated fatty acid usually lowers the melting point.

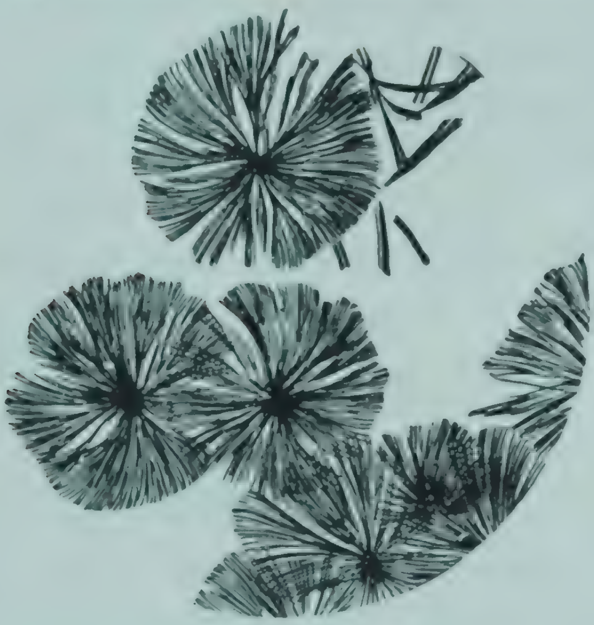


FIG. 34. BEEF FAT.
(Long)

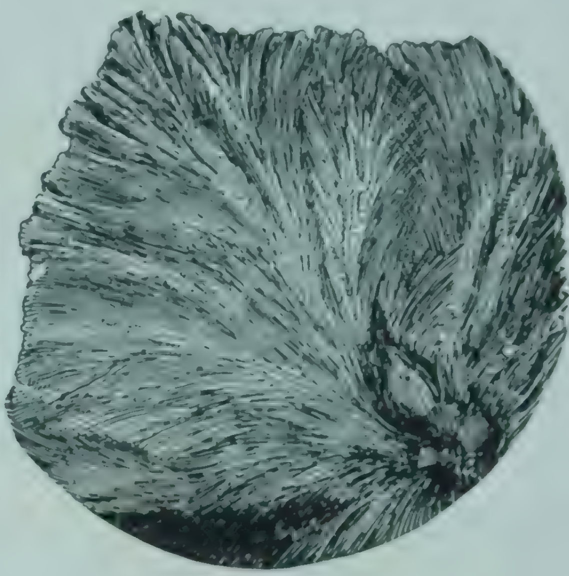


FIG. 35. MUTTON FAT.
(Long)

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[2] Mattson, Benedict, Martin, and Beck: *J. Nutrition*, **48**, 335 (1952).

[3] Zechmeister, in Schönfeld: *Chemie und Technologie der Fette und Fettprodukte*, Vol. I, Vienna, Springer, 1936, pp. 149–193.

Although most fats may exhibit sharp melting points under certain specified conditions, marked differences can be demonstrated between melting point and solidifying point. These discrepancies have been related to the fact that fats may occur in several polymorphic forms, depending upon the rate of cooling and the temperature at which they are maintained. The several forms are referred to as α, β, β', and γ types. They vary in stability, melting point, gross and microscopic appearance, x-ray diffraction pattern, crystal structure, density, and other properties. The γ form has the lowest melting point; the α, β' (when present) and β forms have progressively higher melting points. The polymorphic forms change to the β isomorph on standing; this is the most stable of the polymorphic forms. Ferguson and Lutton[4] reviewed the polymorphism of fats, and the melting points of the known modifications have been listed by Bailey.[5]

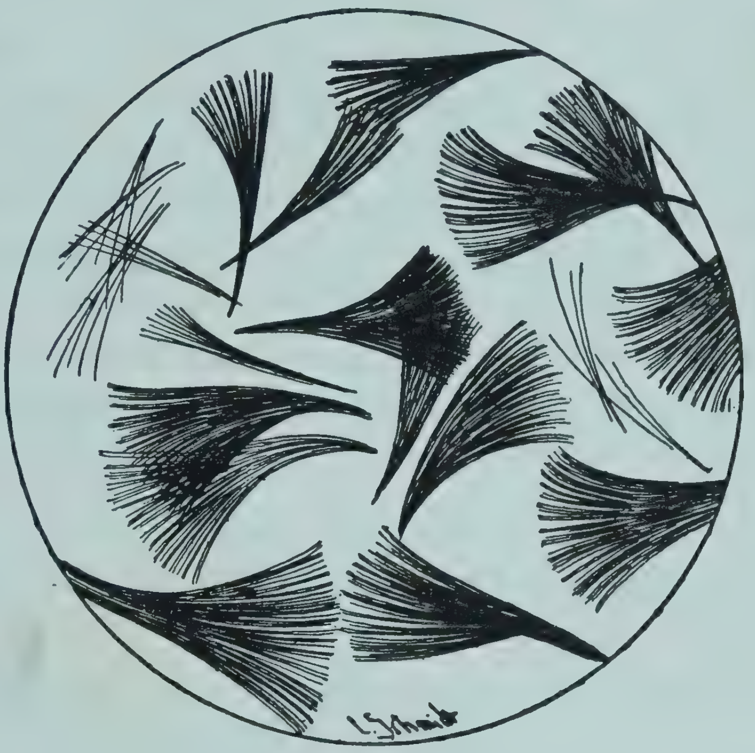

FIG. 36. PORK FAT.

Mixed glycerides containing a high proportion of unsaturated fatty acids are usually liquid at room temperature, as indicated above, and are commonly called oils. These oils take up hydrogen at their double bonds, in the presence of catalysts such as finely divided nickel, and are thus converted into solid fats. This process is called *hardening* or *hydrogenation*. Many commercial fats are partially hydrogenated vegetable oils. There is no known nutritional objection to the use of properly hydrogenated vegetable oils in place of animal fats. For example, in experiments on rats and on man it was shown that hydrogenated vegetable oil was as satisfactorily digested and utilized as lard, and was less liable to cause gastric or intestinal symptoms of an objectionable nature.[6] Furthermore, under conditions in which oxidation may cause destruction of essential factors in the diet, the use of hydrogenated oil is claimed to be preferable to the use of unhydrogenated vegetable or animal fats.[7]

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[4] Ferguson and Lutton: *Chem. Revs.*, **29**, 355 (1941).

[5] Bailey: *Melting and Solidification of Fats*, New York, Interscience Publishers, Inc., 1951.

[6] Smith, Miller, and Hawk: *J. Biol. Chem.*, **23**, 505 (1915); Smith: *Ohio State Med. J.*, **39**, 425 (1943); Deuel and Greenberg: *Fortschr. Chem. Org. Naturstoffe*, **6**, 1 (1950).

[7] Miller: *J. Nutrition*, **26**, 43 (1943).

When a fat is allowed to stand for a sufficient length of time in contact with air and moisture, particularly in the presence of light and heat, certain changes occur and it becomes rancid. Three types of rancidity have been described; namely, oxidative, ketonic, and hydrolytic. The first is the most important in the spoilage of fat. Rancid fats are unpalatable and appear to be slightly toxic for some individuals and destructive to other factors in the food such as carotene and vitamin A[8] and vitamin E.[9] The preservation of a mixed food is frequently a matter largely of the prevention of fat deterioration.

Prevention of Rancidity by Antioxidants. Compounds which retard the rate of development of rancidity in fats and oils are known as *antioxidants*. It is now generally agreed that oxidative rancidity proceeds by virtue of a chain reaction. The unsaturated fat first combines with oxygen to form a peroxide. This intermediate either spontaneously breaks down to an aldehyde or forms such a compound after interaction with water. The aldehyde in turn autoxidizes to a peracid, which oxidizes new, unsaturated linkages simultaneously with its transformation to an ordinary acid.

Antioxidants are able to disrupt this chain reaction. Thus after the oxidant (A) is changed to a peroxide (AO_2), the antioxidant (B) behaves as follows:

$$\begin{gathered} AO_2 + B \rightarrow AO + BO \\ AO + BO \rightarrow A + B + O_2 \end{gathered}$$

By this series of changes the chain reaction is broken, the original unsaturated acid is restored, and the antioxidant is free to continue its protective action against oxidation at the double bonds. The antioxidants are active even in traces; one molecule of hydroquinone will protect 40,000 molecules of acrolein from oxidation.

Ordinarily, vegetable fats are much more resistant to spontaneous oxidation than are animal fats. This resistance is due to the presence in the former of natural antioxidants, referred to as *inhibitols*. Tocopherols (vitamins E) are the most effective antioxidants, the δ isomer being the most and the α isomer the least effective of the several naturally occurring tocopherols. Fats can be protected from spoilage by the use of a number of natural and artificial compounds, which include phenols and hydroquinones, NDGA (nordihydroguaiaretic acid), gum guaiac, propyl gallate, gallic acid, tannins and tannic acid, butylhydroxyanisole, and other related compounds. The effectiveness of the so-called inhibitols is markedly augmented by the presence of synergistic agents such as phosphoric acid, ascorbic acid, citric acid, and certain amino acids. *Prooxidants*, on the other hand, are substances which increase the susceptibility of fats to oxidation. These include certain carotenoids and chlorophyll, as well as copper and iron salts. Cyanide and the various acids just mentioned act as *antiprooxidants* in the sense that they "sequester" the oxidative metallic ions.

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[8] Lease, Weber, and Steenbock: *J. Nutrition*, **16**, 571 (1938).

[9] Fitzhugh, Nelson, and Calvery: *Proc. Soc. Exptl. Biol. Med.*, **56**, 129 (1944).

Emulsions. When oil or liquid fat is shaken with water, it becomes finely divided and is dispersed in the water to form what is known as an emulsion. Emulsification with water alone is of course inefficient and transitory. In the presence of *emulsifying agents* dispersion of fat globules is more complete and hence more permanent. Among the important emulsifying agents are soaps, proteins, bile salts, mono- and diglycerides, and such technologically useful substances as the fatty acid esters of polymeric glycols or sugar alcohols like Myrj, Span, Tween, etc. Emulsifying agents act by lowering the surface tension of the aqueous phase and are presumably adsorbed on the surface of the tiny oil globules to form a film which minimizes the tendency of the globules to coalesce. A very fine powder which is wet by oil but not by water will likewise form a film around oil droplets and stabilize the emulsion. Oil-in-water emulsions are also stabilized by the use of gums which increase the viscosity of the aqueous or external phase. The formation of emulsions is of importance in pharmacy, in industrial practice, and in the processes of fat digestion in the intestinal tract.

Hydrolysis of Fats. Fats may be hydrolyzed by various agents, with the liberation of fatty acids and glycerol. Such agents are superheated steam, long-continued action of air and light, bacteria, and the enzyme lipase. When boiled with alkali the fats give glycerol and the metallic salt of the fatty acid.

$$\underset{\textbf{Tripalmitin}}{C_3H_5(OOCC_{15}H_{31})_3} + 3NaOH \rightarrow \underset{\textbf{Glycerol}}{C_3H_5(OH)_3} + \underset{\textbf{Sodium palmitate}}{3C_{15}H_{31}COONa}$$

This process is known as saponification, and the metallic salts of the higher fatty acids are known as soaps. The ordinary hard soaps of commerce are chiefly sodium soaps. Potassium forms soft soaps, of which green soap is an example. Calcium and magnesium form insoluble soaps. When ordinary soap is added to hard water a certain amount is used up in the precipitation of calcium and magnesium salts before the soap becomes effective. Lead salts also form insoluble soaps.

The detergent (cleansing) action of soaps is due largely to their ability to lower surface tension and thus facilitate emulsification of oily or greasy material, which can then be washed away. The commercial production of synthetic detergents has become of importance; these may be exemplified by one type which is a compound of sulfuric acid and cetyl alcohol, $C_{16}H_{33}OH$, the alcohol analogue of palmitic acid. Synthetic detergents act similarly to soaps by lowering surface tension; they are not inactivated by calcium and magnesium, however, and are therefore equally effective in hard and soft water, as the ordinary soaps are not.

Methods Used in Study of Fats. As the separation and identification of individual fats is a difficult matter, the properties commonly studied are of a general character. The *saponification value* of a fat is the number of milligrams of KOH required to neutralize the free or combined fatty acid in 1 g. of fat. It is determined by saponification and titration of excess alkali and is a measure of the mean molecular weight of the fatty acids present in the fat. The *Reichert-Meissl* number of a fat is a measure of the amount of volatile fatty acids and is determined by titration of the steam distillate. The volatile fatty acids, which also include the soluble ones, are those containing ten or fewer carbon atoms. Butter is rich in these, but most butter substitutes are not. The determination of these acids is of use in detecting adulteration of butter.

The unsaturated fatty acids, of which oleic acid is an example, differ from the saturated acids in their power to take up iodine at the double bond. $—CH{=}CH— + I_2 \rightarrow —CHI—CHI—$. The *iodine value* is thus a measure of the amount of unsaturated fatty acid present in a fat. The more highly unsaturated fatty acids (di-, tri-, and tetraethenoid) are determined by their spectrophotometric absorption characteristics.

Waxes. The waxes are esters of fatty acids with monatomic alcohols. Examples are spermaceti, containing chiefly the palmitate of cetyl alcohol ($C_{16}H_{33}OH$), and beeswax, consisting mainly of the palmitate of myricyl alcohol ($C_{30}H_{61}OH$). They are saponified with greater difficulty than fats and are not attacked by lipase.

Compound Lipides and Sterols. See Chapter 11, Nervous Tissue and Chapter 18, Bile and Liver Function.

Biological Importance of Fats. Fats serve as a storage food in plant and animal organisms, and are of great importance for cellular processes, evidently along with other lipides. They are constituents of cell membranes, and are thus concerned with the phenomena of cell permeability and cell organization. The highly unsaturated fatty acids, linoleic and linolenic, appear to be necessary in the diet of certain animals to prevent a skin syndrome. (See Chapter 33 for a further discussion of fats and fat metabolism.)

EXPERIMENTS ON FATS

1. *Fat Solvents.* Test the solubility of olive oil in water, dilute acid, and alkali; and in cold alcohol, hot alcohol, benzene, chloroform, ether, and carbon tetrachloride. (Cottonseed, soybean, or peanut oil may be used in place of olive oil in all the tests in this section.) Which are the best solvents for fats?

2. *Formation of a Translucent Spot on Paper.* Place a drop of olive oil upon a piece of ordinary writing paper. Note the semitransparent appearance of the paper at the point of contact with the fat.

3. *Reaction.* Try the reaction of fresh olive oil to litmus or other suitable indicator paper. (Moisten the paper with distilled water before making the test.) Repeat the test with rancid olive oil.[10] What is the reaction of a fresh fat and how does this reaction change upon allowing the fat to stand for some time?

4. *Formation of Acrolein from Olive Oil.* (a) Place about 0.5 inch powdered potassium bisulfate ($KHSO_4$) in a clean, dry test tube. Drop no more than 3 or 4 drops of olive oil on the salt and heat, cautiously at first, and then more strongly. Note the irritating odor of acrolein. The glycerol of the fat is dehydrated and acrylic aldehyde or acrolein is produced. This is the reaction which takes place:

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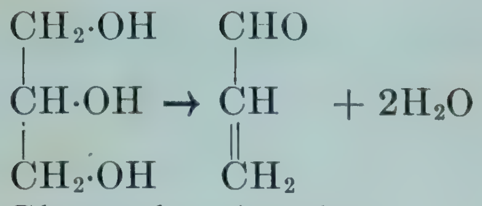
[10] To prepare rancid olive oil add 5 drops of oleic acid to 10 ml. of olive oil. Mix well.



$$\begin{array}{lcll} CH_2{\cdot}OH & & CHO & \\ | & & | & \\ CH{\cdot}OH & \rightarrow & CH & + 2H_2O \\ | & & \| & \\ CH_2{\cdot}OH & & CH_2 & \end{array}$$

Glycerol **Acrolein**

(b) Repeat the above test using (1) 3 to 4 drops of oleic acid and (2) a little solid carbohydrate instead of olive oil. Heat vigorously and note the odor of SO_2 in both tubes. Organic compounds reduce $KHSO_4$ to SO_2, which is often mistaken for acrolein. Even fats, if heated too vigorously, yield SO_2 as well as, or after, the acrolein.

5. *Emulsification.* (a) Shake up a drop of neutral[11] olive oil with a little water in a test tube. The fat becomes finely divided, forming an emulsion. This is not a permanent emulsion since the fat separates and rises to the top upon standing.

(b) To 5 ml. of water in a test tube add 2 or 3 drops of 0.5 per cent Na_2CO_3. Introduce into this faintly alkaline solution a drop of neutral olive oil and shake. The emulsion, while not permanent, is not so transitory as in the case of water free from sodium carbonate.

(c) Repeat (b), using rancid olive oil. What sort of emulsion results? In this case the alkali combines with the free fatty acid to form soap, and this soap, being an emulsifying agent, emulsifies the fat.

(d) Shake a drop of neutral olive oil with dilute albumin solution. What is the nature of this emulsion? Examine it under the microscope.

(e) Repeat (d), using bile salt solution instead of albumin solution. Compare the stability of the emulsion with that obtained in (a). What is one of the physiological functions of the bile?

6. *Fat Crystals.* Dissolve about 40 drops of melted lard in 10 ml. of ether in a test tube, stopper loosely with some filter paper, and allow the mixture to evaporate spontaneously until crystals begin to separate out. Transfer some of the material to a slide, examine the crystals under the microscope, and compare them with those reproduced in Figs. 34, 35, and 36.

7. *Saponification of Bayberry Tallow.*[12] Place about 10 g. of bayberry tallow in a 600-ml. beaker; add about 150 ml. of distilled water and 50 ml. of 10 per cent potassium hydroxide solution (not sodium hydroxide). Boil the mixture for 10 to 15 minutes, or until saponification is complete (no oily layer remaining on the surface). When saponification is complete,[13] remove 25 ml. of the hot soap solution, dilute it with about 100 ml. of distilled water, and reserve this solution for use in Exp. 8 and 9 below. To the remainder of the hot solution slowly add concentrated hydrochloric acid until the mixture is acid (about 10 ml. are required).[14] The free fatty acid formed will rise to the top as a clear oily layer. Now cool the solution, permitting the fatty acid to solidify and form a cake. In this instance the fatty acid is principally palmitic

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[11] *Neutral* olive oil may be prepared by shaking ordinary olive oil with a 10 per cent solution of sodium carbonate. This mixture should then be extracted with ether and the ether removed by evaporation. The residue is neutral olive oil.

[12] Bayberry tallow is derived from the fatty covering of the berries of the wax myrtle. It is therefore frequently called *myrtle wax* or *bayberry wax*.

[13] Place 1 or 2 drops in a test tube full of hot distilled water. If saponification is complete, the soap formed will remain in solution and no oil will separate out.

[14] Under some conditions a purer product is obtained if the soap solution is cooled before precipitating the fatty acid.

acid. Remove the cake, break it into small pieces, wash it with water by decantation, and place in a beaker containing about 50 ml. of 95 per cent alcohol. Warm the mixture by placing the beaker in a vessel containing some hot water, until the palmitic acid is dissolved; then filter through a dry filter paper and allow the filtrate to cool slowly in order to obtain satisfactory crystals.

When the palmitic acid has completely crystallized, filter off the alcohol, dry the crystals between filter papers and by exposure to the air, and try the tests given in Exp. 11. Write the reactions which have taken place in this experiment.

8. Salting-out of Soap. To 100 ml. of soap solution, prepared as described above, add solid sodium chloride to the point of saturation, with continual stirring. (If the soap has solidified due to cooling, dissolve it by warming before adding the salt.) The soap, which is in colloidal solution, is precipitated when its solution is saturated with sodium chloride. Why?

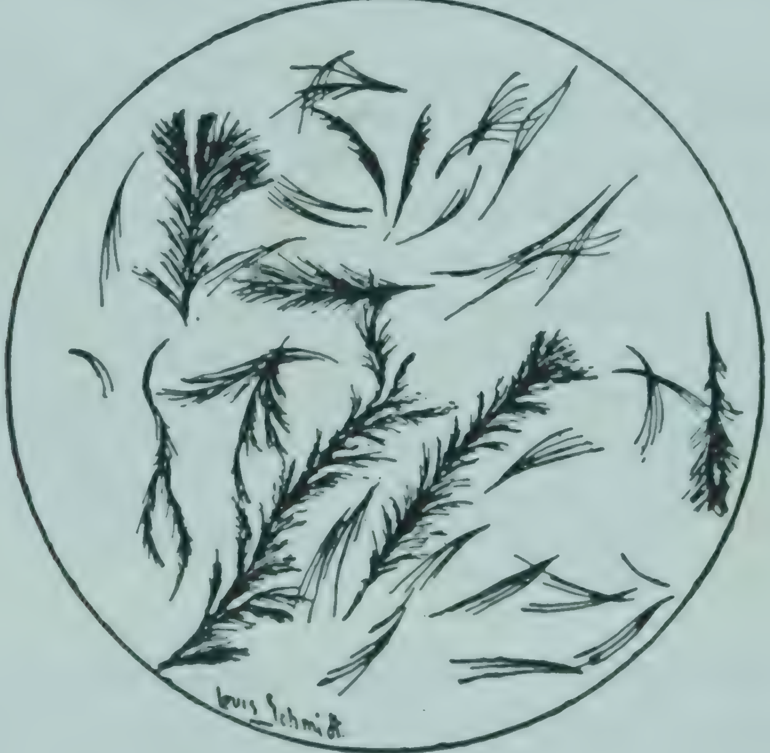

Fig. 37. Palmitic Acid.

9. Formation of Insoluble Soaps. Introduce 5 ml. of soap solution into each of two test tubes. To the contents of one tube add a small amount of a solution of calcium chloride and to the contents of the other tube add a small amount of a solution of magnesium sulfate. Note the formation of insoluble soaps of calcium and magnesium.

10. Surface Tension of Soap Solutions. Determine the *drop number* of the soap solution as compared to pure water, as described on p. 23. What is the approximate surface tension of the soap solution relative to that of water? Is a drop of a soap solution equal in volume to a drop of an ordinary aqueous solution?

11. Palmitic Acid. (a) Microscopic: Examine the crystals under the microscope and compare them with those shown in Fig. 37.

(b) Solubility. Test the solubility of palmitic acid in the same solvents as used on fats (see p. 106).

(c) MELTING POINT. Determine the melting point of palmitic acid by any standard method. A value lower than the theoretical will be obtained because of the presence of impurities.

(d) FORMATION OF TRANSLUCENT SPOT ON PAPER. Melt a little of the fatty acid and allow a drop to fall upon a piece of ordinary writing paper. How does this compare with the action of a fat under similar circumstances?

(e) ACROLEIN TEST. Apply the test as given under 4, p. 106. Explain the result.

(f) IODINE ABSORPTION TEST. For directions see Exp. 14.

12. Saponification of Lard. To 25 g. of lard in a flask add 75 ml. of alcoholic-potash solution[15] and warm on a water bath until saponification is complete. (This point is indicated by the complete solubility of a drop of the solution when allowed to fall into a little distilled water.) Now transfer the solution from the flask to an evaporating dish containing about 100 ml. of water and heat on a water bath until all the alcohol has been driven off. Acidify the solution with hydrochloric acid and cool. Remove the fatty acid which rises to the surface,[16] neutralize the solution with sodium carbonate, and evaporate to dryness. Extract the residue with alcohol, remove the alcohol by evaporation on a water bath, and on the residue of glycerol thus obtained make the tests as given below.

13. Glycerol. (a) TASTE. What is the taste of glycerol?

(b) SOLUBILITY. Try the solubility of glycerol in water, alcohol, and ether.

(c) ACROLEIN TEST. Repeat the test as given under 4, p. 106, using 2 drops of glycerol.

(d) BORAX FUSION TEST. Fuse a paste of glycerol and powdered borax on a platinum wire and note the characteristic green flame. This color is due to the glycerol ester of boric acid.

(e) BENEDICT'S TEST. Add a few drops of glycerol to 5 ml. of Benedict's reagent and boil for 2 to 3 minutes. How does the result compare with the results on the sugars?

(f) SOLUTION OF $Cu(OH)_2$. Form a little cupric hydroxide by mixing copper sulfate and sodium hydroxide. Add a little glycerol to this suspended precipitate and note what occurs. Explain.

14. Iodine Absorption Test. Dissolve 5 to 10 drops of an unsaturated organic acid, e.g., oleic acid, in about 5 ml. of chloroform. Add some Hübl's iodine solution,[17] a drop or two at a time, and shake between additions. The solution will be decolorized if unsaturated acids are present. This is due to the absorption of the iodine. The test should be controlled by shaking chloroform and iodine solution to which no acid has been added.

15. Determination of Saponification Number. Measure 5 ml. of oil into a 250-ml. Erlenmeyer flask with a clean dry pipet. Calculate the weight of the oil from the specific gravity. (The specific gravity of olive oil is 0.916.) (Direct weighing of the oil is more accurate.) Add 50 ml. of alcoholic 0.5 N KOH (made up in 90 per cent alcohol) from a pipet. To another flask not containing oil add a similar 50-ml. portion of the KOH. Fit each flask with a reflux air condenser and boil by immersion in a large beaker of boiling water for half an hour with frequent agitation. Add to each flask 1 ml. of 1 per cent alcoholic

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[15] A 20 per cent solution in 40 per cent alcohol may be used.

[16] After drying the acid make an iodine absorption test as described in Exp. 14.

[17] See Appendix.

solution of phenolphthalein and titrate with 0.5 N HCl. Subtract the titration value of the control from that of the unknown. One ml. of the HCl is equivalent to 0.02805 g. of KOH. Calculate the number of mg. of KOH required to saponify 1 g. of the oil.

16. Determination of Iodine Absorption Number. **With a clean dry pipet measure 0.3 ml. of oil into a dry 100-ml. Erlenmeyer flask. Calculate the weight of the oil from the specific gravity. (For accurate work the oil must be weighed.) Add 10 ml. of carbon tetrachloride and after solution of the oil add exactly 25 ml. of Wijs' iodine solution.[18] Mix well, stopper, and put in a dark place for 1 to 2 hours. Transfer quantitatively to a 500-ml. flask, washing out the small flask with 10 ml. of 10 per cent KI solution and with water to make a volume of about 250 ml. Titrate with thiosulfate to a light brown color, add starch paste and titrate to disappearance of the blue color. After the blue color has disappeared from the aqueous phase, the carbon tetrachloride layer in the bottom of the flask usually contains untitrated iodine as evidenced by a pink or violet color. This iodine may be brought into the aqueous layer by continuous shaking. The end point of the titration is reached when both the aqueous and nonaqueous phases are completely colorless. Calculate the number of centigrams of iodine absorbed by 1 g. of fat. This is the iodine absorption number, or the iodine value, of the oil.**

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[18] See Appendix.

4

Proteins: Their Composition and Hydrolysis; Amino Acids

PROTEINS

Definition. Proteins have been defined as extremely complex nitrogen-containing organic compounds which are found in all animal and vegetable cells, where they constitute a major part of the living protoplasm of those cells. The Dutch physiological chemist G. J. Mulder, who at the suggestion of the famous chemist, Berzelius, derived the word protein from the Greek πρῶτος, "first," said in 1840: "In both plants and animals a substance is contained which is produced within the former, and imparted through the food to the latter. It is one of the most complicated substances, is very changeable in composition. . . . It is unquestionably the most important of all known substances in the organic kingdom. Without it no life appears possible on our planet. Through its means the chief phenomena of life are produced." Proteins, carbohydrates, and fats form the three great classes of foodstuffs, but the function of protein in the diet is not primarily to supply energy, as is true of the other two, but to furnish certain essential components of the living tissue of the organism itself. Although plants, including many bacteria, are capable of synthesizing proteins from simple organic and inorganic nitrogenous compounds, this ability has been lost to such an extent by the higher animals that they must depend upon preformed proteins or rather certain of their specific degradation products, the α-amino acids, for the continuance of life.

The capacity of the living organism for storing proteins is limited and relatively small when compared to its capacity for storing carbohydrates and fats. Proteins are, however, stored under special conditions as in eggs and seeds for use by the developing or immature organism until it can obtain food from its environment. Although the carbohydrates and lipides are both essential constituents of the colloidal complex which we call protoplasm, the proteins are of paramount importance not only because of their peculiar chemical and physicochemical properties but also because they appear to confer upon various types of cells their biological specificity. In the main, identical lipides and carbohydrates may be found in cells of both plants and animals of widely different species; the proteins, however, are usually highly characteristic of the species of plant or animal, and, more often than not, of the specific organ in which they are found.

Composition. The proteins differ from carbohydrates and fats not only in their function in the organism, but also in elementary composition.

In addition to carbon, hydrogen, and oxygen, the proteins invariably contain nitrogen and generally also sulfur. The percentage composition of the large number of proteins from different sources which have been studied falls between the following rather narrow limits: C = 50 to 55 per cent, H = 6.0 to 7.3 per cent, O = 19 to 24 per cent, N = 13 to 19 per cent, S = 0 to 4 per cent. Proteins have also been described which contain phosphorus, iron, copper, iodine, manganese, zinc, and other elements. None of these latter elements, with the exception of iodine, has thus far been found as a constituent of the α-amino acids, the fundamental units from which proteins are built up by the organism, and until we have more exact information concerning the composition and structure of the protein molecule, we may assume that these elements are combined with protein in some unknown way, or, as has been shown in the case of iron, that they are constituents of nonprotein substances which are combined with protein in a way which confers new and characteristic properties upon the complex formed.

General Properties. The almost infinite number of proteins which are present in nature give certain reactions which serve to identify them as a distinct class of biological substances. Almost all soluble proteins form colloidal solutions. This indicates either that the molecules of protein are so large that even when dispersed in molecular solution they have the properties of size and surface which characterize colloidal particles, or else that there is some force connected with protein molecules which predisposes them to form aggregates of colloidal size. There are good reasons for believing that the colloidal properties of protein solutions are due in some cases to the first and in other cases to the second of these possibilities. The colloidal solutions formed by proteins are of the emulsoid type, thus requiring large concentrations of electrolytes for precipitation. The proteins give certain color reactions which, however, are not specific for the protein molecule as such but for various chemical groups which it is thus shown to contain. Although proteins generally precipitate from their solutions in an amorphous condition, many of them have been isolated from both animal and vegetable sources in the form of characteristic crystals. The proteins are precipitated from their solutions by salts of heavy metals, such as silver nitrate, lead acetate, etc.; by acids such as tannic acid, phosphotungstic acid, metaphosphoric acid, etc.; and by many dyes and detergents. These precipitation reactions are probably due, in part at least, to the formation of insoluble compounds of the protein and the precipitating agent and in part to the colloidal character of protein solutions. The proteins exhibit amphoteric properties, combining with both acids and bases to form ionizable salts. Finally, upon complete hydrolysis the proteins yield crystalline substances of definite composition belonging to the class of compounds known as α-amino acids.

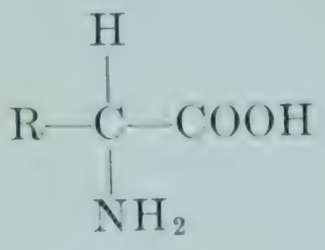
Investigation of Protein Composition. The constancy in elementary composition of proteins from widely differing sources and the great individual differences in chemical and physical properties preclude elementary analysis as a method of characterizing individual proteins. There are, however, four main lines of attack used in the investigation of the protein molecule: (1) Complete hydrolysis of the protein into its constituent units, the α-amino acids; (2) partial hydrolysis to yield relatively small degradation products (di- and tripeptides, etc.) which are capable of separation and complete characterization; (3) stepwise degradation, that is, removing one amino acid after another from one end of the polypeptide chain; and (4) the study of the physicochemical, colloid-chemical, and biological reactions of the completely intact molecule as well as of the slightly changed (denatured) molecule. Until comparatively recently the method of complete hydrolysis was the one most commonly employed. This procedure is of great importance since it yields the individual units from which the protein is formed. However, just as elementary analysis of the less complex organic molecules tells us the percentage composition of the substance under examination without indicating the atomic arrangement within the molecule, so complete hydrolysis of a protein reveals the quantities of the various constituent amino acids without giving much indication as to their special arrangement within the giant molecule. In order to reveal the great individual differences among proteins and to gain an insight into their specific biological functions, such as the hormonal action of insulin, the enzymatic effects of pepsin, the role of myosin in muscle contraction, the pathogenicity of tobacco mosaic virus, etc., all four modes of study must be used. The work of the chemist, biologist, physicist, and analyst must be known and appreciated by each member of the team before the problem of the complete structure of even one protein is to be solved.

Hydrolysis. Hydrolysis of proteins may be effected: (1) By boiling with mineral acids or strong alkalies, at atmospheric or increased pressures, (2) by treatment with certain long-chain sulfonic acids (cetylsulfonic acid, diphenylbenzenesulfonic acid, etc.), and (3) by digestion with proteolytic enzymes. Commonly, hydrolysis of a protein is carried out by boiling it with five to ten times its weight of 6 N hydrochloric acid or 8 N sulfuric acid for 6 to 24 hours. Under special circumstances other reagents, such as hydriodic acid, oxalic acid, 5 N sodium hydroxide, hot saturated barium hydroxide, or a mixture of formic and hydrochloric acids, can be used. Acid hydrolysis, especially if the protein contains carbohydrate, usually results in the complete destruction of the amino acid tryptophan, and may result in the partial decomposition of certain other amino acids. The prolonged heating necessary to effect complete hydrolysis by strong alkalies does not affect tryptophan, but results in the partial or complete destruction of cysteine, cystine, and arginine, and in the racemization and consequent loss of optical activity of all the amino acids. Enzymatic hydrolysis has none of these disadvantages of acid or alkaline hydrolysis but it is very time-consuming and is seldom complete. Hydrolysis of proteins with the above-mentioned sulfonic acids requires further investigation.

Hydrolysis of the protein molecule by any of the methods above leads to the formation of a series of ill-defined fragments of decreasing complexity known as proteoses, peptones, and polypeptides, the final products being amino acids. Certain amino acids are split off early in the hydrolytic process. This liberation of amino acids continues until the larger intermediate fragments have all been reduced to these simpler compounds. Physically the hydrolysis of proteins consists in a breaking down of the large, colloidal, nondiffusible complexes into a series of fragments in which the colloidal character becomes less and less pronounced, until finally only the simple, crystalloidal, and diffusible amino acids remain. Since the amino acids represent, therefore, the "building stones" of the protein molecule, we may well begin the study of that molecule by a consideration of the structure and reactions of these fundamental units.

AMINO ACIDS

The amino acids thus far isolated from protein hydrolyzates are α-amino acids—that is, they have an amino (NH_2) group attached to the same carbon atom that holds the carboxyl (COOH) group. Their general formula[1] is therefore



$$R-\overset{\displaystyle H}{\underset{\displaystyle NH_2}{\overset{|}{\underset{|}{C}}}}-COOH$$

Individual amino acids differ in the character of the radical R attached to the α carbon atom.

CLASSIFICATION

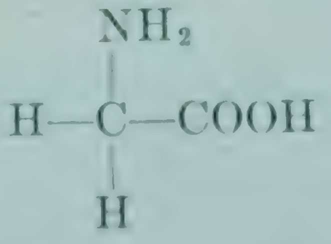
The amino acids may be conveniently classified according to the number of their amino and carboxyl groups, as follows: (1) Neutral amino acids, containing one amino and one carboxyl group, (2) acidic amino acids, containing an excess of carboxyl groups, and (3) basic amino acids, containing an excess of basic nitrogen. The amino acids in each group may be further subdivided according to whether the radical R in the general formula represents an aliphatic, aromatic, or heterocyclic nucleus.

NEUTRAL AMINO ACIDS

These acids are often referred to as monoamino-monocarboxylic acids because they contain one amino and one carboxyl group. Solutions of these acids react essentially neutral. These acids form the largest group in the protein molecule and most of them may be separated in one fraction from the products of protein hydrolysis. Because of their great chemical and physical similarities, the isolation of the individual acids in this fraction presents experimental difficulties.

A. ALIPHATIC AMINO ACIDS

1. Glycine, $C_2H_5O_2N$ (aminoacetic acid)



$$H-\overset{\displaystyle NH_2}{\underset{\displaystyle H}{\overset{|}{\underset{|}{C}}}}-COOH$$

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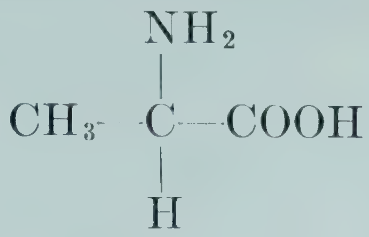
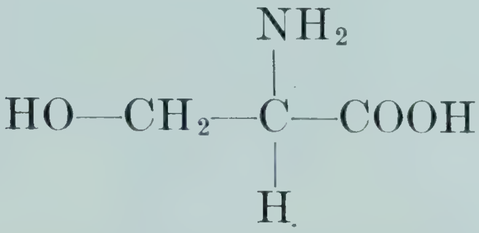
[1] The only exceptions to this general formula are found with the two amino acids proline and hydroxyproline (*q.v.*), in which the α-nitrogen is part of a cyclic structure.

2. Alanine, $C_3H_7O_2N$ (α-aminopropionic acid)



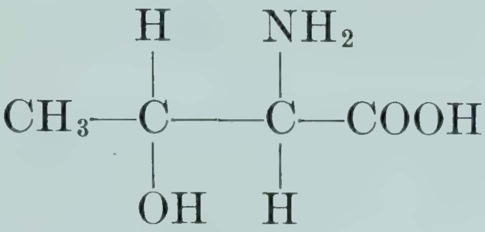
$$CH_3-\overset{NH_2}{\underset{H}{C}}-COOH$$

3. Serine, $C_3H_7O_3N$(β-hydroxy-α-aminopropionic acid or β-hydroxyalanine)



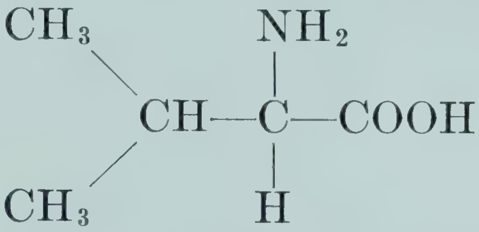
$$HO-CH_2-\overset{NH_2}{\underset{H}{C}}-COOH$$

4. Threonine, $C_4H_9O_3N$ (α-amino-β-hydroxy-*n*-butyric acid)

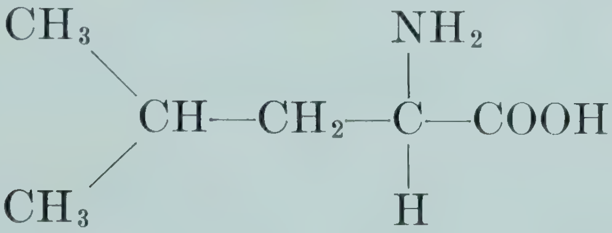


$$CH_3-\overset{H}{\underset{OH}{C}}-\overset{NH_2}{\underset{H}{C}}-COOH$$

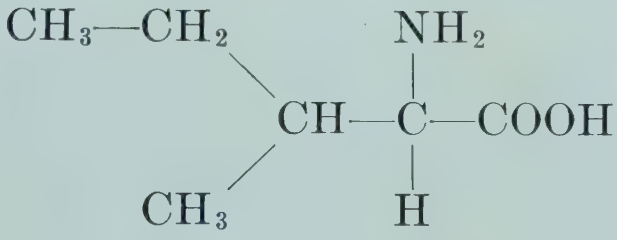
5. Valine, $C_5H_{11}O_2N$ (α-aminoisovaleric acid or β,β-dimethylalanine)



6. Leucine, $C_6H_{13}O_2N$ (α-aminoisocaproic acid or β-isopropylalanine)

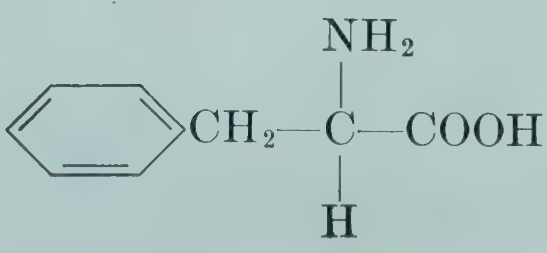


7. Isoleucine, $C_6H_{13}O_2N$ (β-methyl-α-aminovaleric acid or β-methyl-β-ethylalanine)

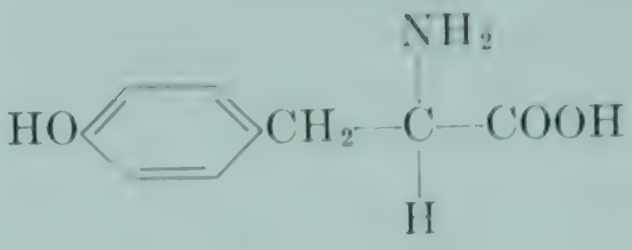


B. AROMATIC AMINO ACIDS

8. Phenylalanine, $C_9H_{11}O_2N$ (β-phenyl-α-aminopropionic acid or β-phenylalanine)

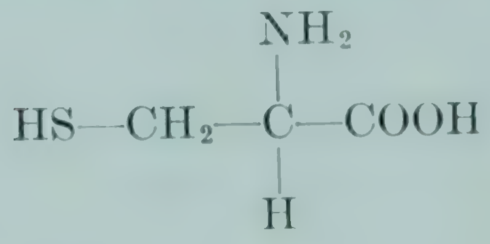


9. Tyrosine, $C_9H_{11}O_3N$ (β-parahydroxyphenyl-α-aminopropionic acid or β-parahydroxyphenylalanine)



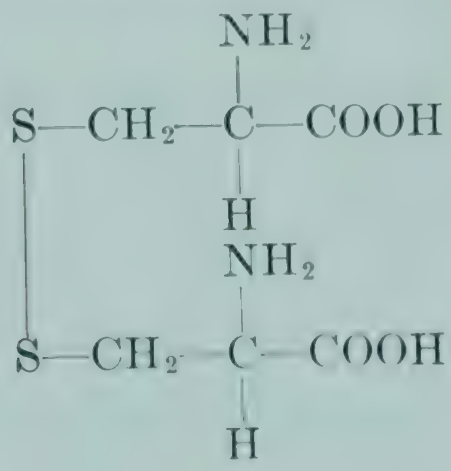
C. SULFUR-CONTAINING AMINO ACIDS

10. Cysteine, $C_3H_7O_2NS$ (β-thiol-α-aminopropionic acid)



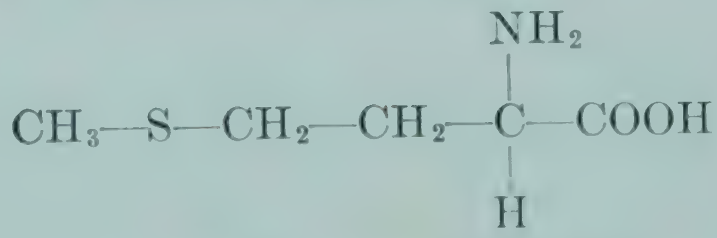
$$\mathrm{HS-CH_2-\underset{H}{\overset{NH_2}{C}}-COOH}$$

11. Cystine,[2] $C_6H_{12}O_4N_2S_2$ (di-(β-thiol-α-aminopropionic acid))



$$\begin{array}{l}\mathrm{S-CH_2-\underset{H}{\overset{NH_2}{C}}-COOH} \\ | \\ \mathrm{S-CH_2-\underset{H}{\overset{NH_2}{C}}-COOH}\end{array}$$

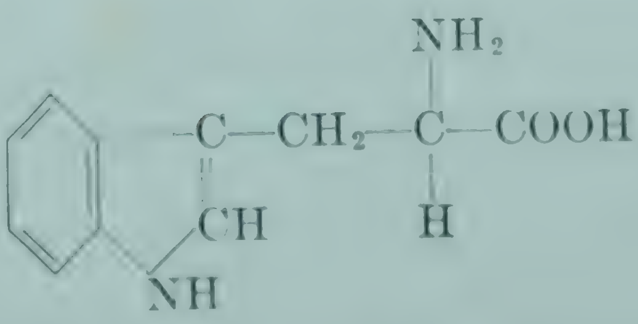
12. Methionine, $C_5H_{11}O_2NS$ (γ-methylthiol-α-amino-*n*-butyric acid)



$$\mathrm{CH_3-S-CH_2-CH_2-\underset{H}{\overset{NH_2}{C}}-COOH}$$

D. HETEROCYCLIC AMINO ACIDS

13. Tryptophan, $C_{11}H_{12}O_2N_2$ (β-3-indole-α-aminopropionic acid or β-indolealanine)



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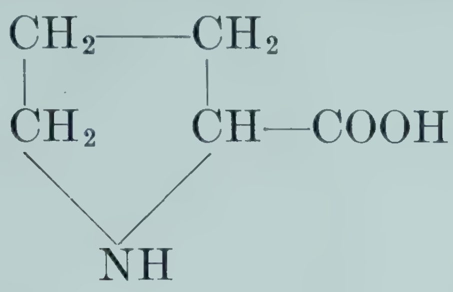
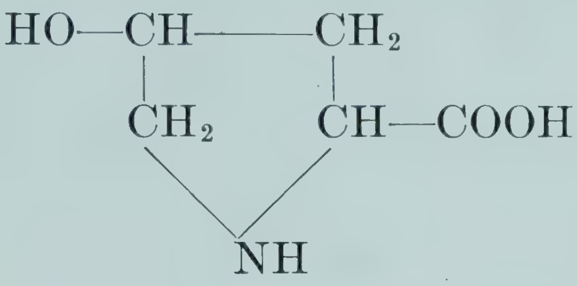
[2] Cystine is included here among the monoamino-monocarboxylic acids because it contains equal numbers of amino and carboxyl groups.

14. Proline, $C_5H_9O_2N$ (pyrrolidine-2-carboxylic acid)



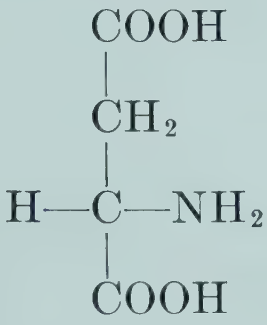
15. Hydroxyproline, $C_5H_9O_3N$ (oxyproline or 4-hydroxypyrrolidine-2-carboxylic acid)



ACIDIC AMINO ACIDS

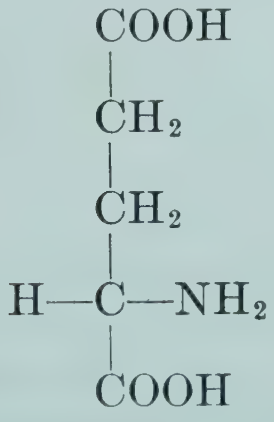
These acids, often referred to as monoamino-dicarboxylic acids, contain more carboxyl than amino groups and are therefore acid in reaction. The acidic nature of glutamic acid and aspartic acid allows their ready separation from the other components of protein hydrolyzates.

16. Aspartic acid, $C_4H_7O_4N$ (α-aminosuccinic acid)



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{H{-}C{-}NH_2} \\ | \\ \mathrm{COOH} \end{array}$$

17. Glutamic acid, $C_5H_9O_4N$ (α-aminoglutaric acid)



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{H{-}C{-}NH_2} \\ | \\ \mathrm{COOH} \end{array}$$

BASIC AMINO ACIDS

These acids are predominantly basic in reaction and are precipitated from protein hydrolyzates by the addition of phosphotungstic acid[3] or they may be selectively adsorbed on weak cation exchange resins.

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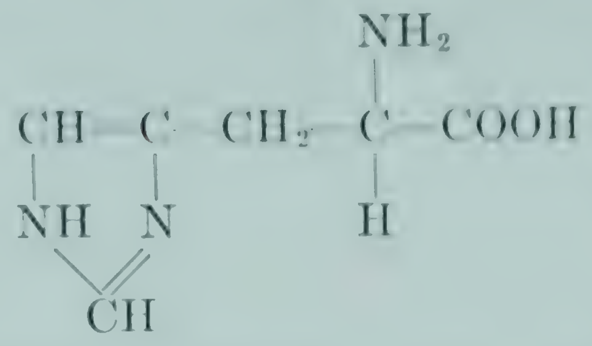
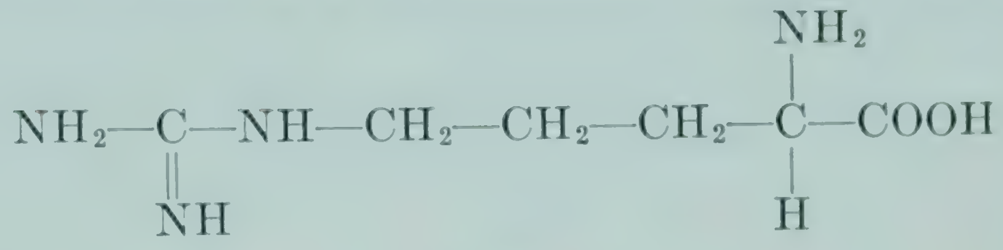
[3] Proline and cystine are also precipitated to a certain extent by phosphotungstic acid.

18. Histidine, $C_6H_9O_2N_3$ (β-imidazole-α-aminopropionic acid or β-imidazolealanine)

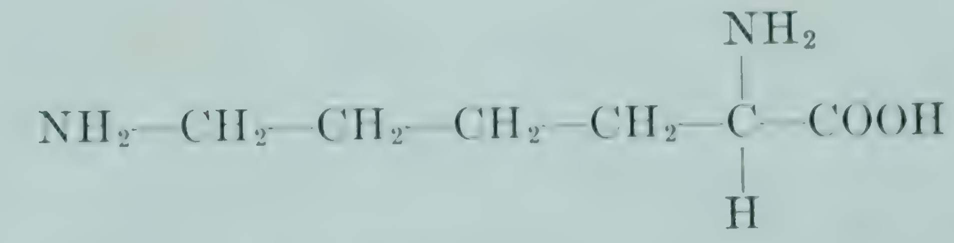


19. Arginine, $C_6H_{14}O_2N_4$ (δ-guanidino-α-aminovaleric acid)



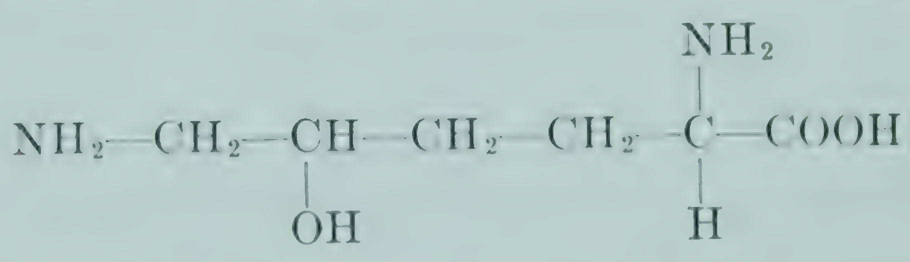
$$\begin{array}{c}\\ NH_2-\underset{\underset{NH}{\|}}{C}-NH-CH_2-CH_2-CH_2-\overset{\overset{NH_2}{|}}{\underset{\underset{H}{|}}{C}}-COOH\end{array}$$

20. Lysine, $C_6H_{14}O_2N_2$ (α-ε-diaminocaproic acid)



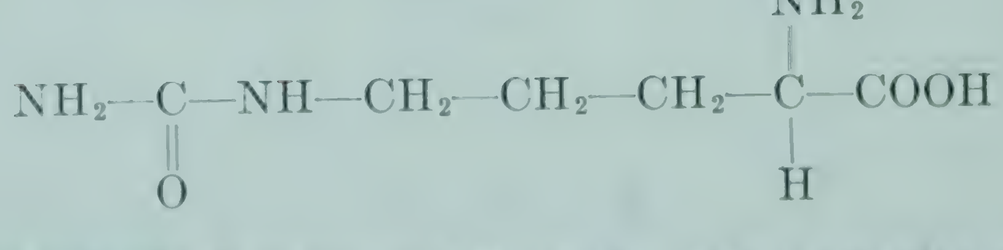
$$NH_2-CH_2-CH_2-CH_2-CH_2-\overset{\overset{NH_2}{|}}{\underset{\underset{H}{|}}{C}}-COOH$$

21. Hydroxylysine, $C_6H_{14}O_3N_2$ (α-ε-diamino-δ-hydroxycaproic acid).



$$NH_2-CH_2-\underset{\underset{OH}{|}}{CH}-CH_2-CH_2-\overset{\overset{NH_2}{|}}{\underset{\underset{H}{|}}{C}}-COOH$$

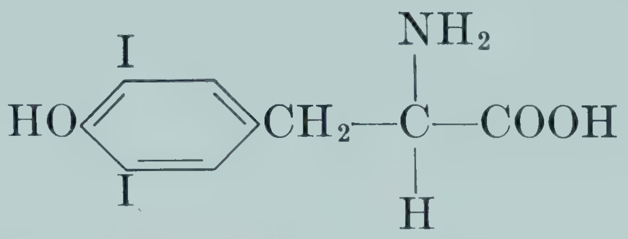
22. Citrulline, $C_6H_{13}O_3N_3$ (δ-carbamino-α-aminovaleric acid)



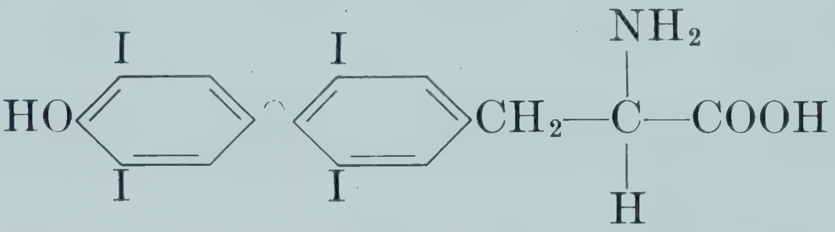
$$NH_2-\underset{\underset{O}{\|}}{C}-NH-CH_2-CH_2-CH_2-\overset{\overset{NH_2}{|}}{\underset{\underset{H}{|}}{C}}-COOH$$

Other Hydrolysis Products of Proteins. Most proteins contain practically all of the amino acids listed above. Occasionally we find that certain amino acids are either entirely absent in a particular protein, or else present in amounts too small to be detected by the available methods of analysis. Thus the protein zein, from maize, contains no lysine or glycine, gelatin yields no tryptophan, and insulin contains no methionine. In other cases we may find that certain specialized proteins contain amino acids not found in any other proteins. The most noteworthy example of this is a globulin obtained from the thyroid gland, thyroglobulin, which normally contains the iodinated tyrosine derivatives, monoiodotyrosine;

3, 5-diiodotyrosine, or iodogorgoic acid; and β-4′-hydroxyphenyl-3′,5′,3, 5-tetraiodotyrosine, or thyroxine. Interestingly enough the bromine analogues of iodotyrosines—namely monobromotyrosine and dibromotyrosine—have been found in a number of Mediterranean corals and Gorgonia.



Diiodotyrosine



Thyroxine

Other Porifera and coelenterates yield monoiodotyrosine, diiodotyrosine, and even traces of thyroxine.

Occasionally substances known as diketopiperazines, which are anhydrides formed by the condensation of two molecules of amino acid, have been found in protein hydrolyzates. It is probable that these are formed during the treatment of the hydrolyzate.

In addition to the above-mentioned amino acids, there are other less well-recognized amino acids in many peptide molecules. Thus α-ϵ-diaminopimelic acid has been found in the insoluble proteins of the microorganism *Corynebacterium diphtheriae;* and iodinated histidine has been reported to be present in pancreatic digests of thyroglobulin.

Acid hydrolysis of proteins also liberates variable quantities of ammonia which come primarily from the hydrolysis of the acid amide groups of asparagine and glutamine, for it is now known that a portion of these dicarboxylic amino acids exists in the peptide chain with the "omega" carboxyl group aminated. A smaller portion of the ammonia presumably comes from the hydrolytic decomposition of certain amino acids such as cysteine, serine, threonine, etc. If alkaline hydrolysis is employed, ammonia is formed from glutamine, asparagine, cystine, cysteine, serine, threonine, and arginine. The last-named substance breaks down to yield ornithine and two molecules of ammonia.

Hydrolysis of proteins also yields small quantities of carbon dioxide, hydrogen sulfide, and free sulfur. These products arise from the destructive decomposition of the more labile amino acids, especially cysteine. Many proteins (serum albumin, egg albumin, thrombin) yield varying amounts of carbohydrate. These carbohydrates appear to be polysaccharides composed of glucosamine and hexose (mannose, galactose). It is not known whether the carbohydrates are an integral part of the protein molecule or whether the protein is composed of a carbohydrate-free portion admixed with a small quantity of a carbohydrate-rich protein (glycoprotein) such as seromucoid, seroglycoid, ovomucoid, etc.

Determination of Amino Acids. The quantitative determination of each of the component amino acids produced during hydrolysis of a protein is a problem of greatest importance since any theory of protein structure must rest ultimately on an exact knowledge of all the units contained in the molecule. This problem is still far from solved since it presents many serious difficulties, not least of which is the preliminary hydrolysis of the protein molecule into its component amino acids without causing decomposition during the hydrolysis.

APPROXIMATE PERCENTAGE COMPOSITION OF SELECTED PLANT PROTEINS*

	Gliadin	*Zein*	*Corn Gluten**	*Wheat Gluten**	*Potato Meal**	*Peanut Meal**	*Soybean Meal**
Nitrogen........	17.66	16.2	12.7	..	..	10.8	7.9
Sulfur..........	1.24	0.52	1.5	..	..	..	..
Arginine........	2.7	1.7	3.1	3.9	5.0	10.6	7.3
Histidine........	2.3	1.3	2.1	2.2	2.2	2.1	2.9
Lysine..........	1.1	0.0	1.5	1.9	8.3	3.0	6.8
Tyrosine........	3.2	5.3	6.3	3.8	2.5	4.4	4.0
Tryptophan.....	0.6	0.1	0.6	0.8	2.1	1.0	1.4
Phenylalanine...	6.9	6.2	6.6	5.5	5.4	5.1	5.3
Cystine.........	2.6	0.8	1.5	2.4	1.3	1.6	1.9
Methionine.....	1.7	2.4	2.5	1.7	2.0	1.0	1.7
Threonine.......	2.1	3.5	4.0	2.5	6.9	1.6	3.9
Serine..........	4.9	8.3	..	4.0	2.6	6.6	4.2
Leucine.........	6.5	23.7	16.0	7.0	9.6	6.7	8.0
Isoleucine.......	5.4	7.3	5.1	4.2	3.7	4.0	6.0
Valine..........	2.7	3.5	5.7	4.1	5.3	4.4	5.3
Glutamic acid...	45.7	26.9	24.5	35.0	7.4	17.4	18.4
Aspartic acid....	1.3	6.6	..	3.8	11.5	15.1	3.7
Glycine.........	<0.5	0.4	4.3	3.3	1.9	5	4.0
Alanine.........	2.1	11.6	..	2.7	6.1	4.2	3.3
Proline.........	13.4	10.5	..	14.1	3.0	5.2	5

* Calculated in g. of amino acid per 16.0 g. of nitrogen.

METHODS. *A. Fractional Distillation of Ethyl Esters.* Fischer in 1901 showed that most neutral amino acids formed esters when their solutions in absolute ethyl alcohol were treated with dry hydrogen chloride. The amino acid esters were separated by fractional distillation *in vacuo*. Using this method, Fischer was able to isolate many of the amino acids in pure crystalline form. The method, however, is very tedious, requires large quantities of protein, and does not yield quantitative results.

B. Van Slyke's Nitrogen-Distribution Method. When an aliphatic primary amine is treated with nitrous acid, its amino nitrogen is quantitatively converted into free nitrogen.

$$R{\cdot}NH_2 + HONO \rightarrow R{\cdot}OH + H_2O + N_2 \uparrow$$

By the use of this reaction, supplemented by other procedures as indicated in the accompanying diagram, Van Slyke developed a method for the determination of the distribution of amino and nonamino nitrogen in the protein together with fairly accurate determinations of cystine, arginine, lysine, and histidine.

DISTRIBUTION OF PROTEIN NITROGEN BY VAN SLYKE METHOD

- *Total Nitrogen* (Kjeldahl determination on portion of hydrolysis mixture)
  - I. *Amide Nitrogen.* (Distilled off as ammonia, after neutralization of hydrolysis mixture with magnesium oxide.)
  - II. *Basic Nitrogen.* (Precipitated by phosphotungstic acid.)
    - a. Cystine. (Contains only amino N. Estimated from sulfur determinations.)
    - b. Lysine. (Contains only amino N.)
    - c. Arginine. (Three-quarters of nitrogen is nonamino N. Half of nitrogen liberated as ammonia by boiling with strong NaOH.)
    - d. Histidine. (Two-thirds of nitrogen is nonamino N.)
  - III. *Monoamino Nitrogen + Nonamino Nitrogen.* (Kjeldahl determination on filtrate from precipitated phosphotungstates.)
    - a. Monoamino N. (Neutral and acid amino acid N. One-half of tryptophan N. Determined by Van Slyke method.)
    - b. Nonamino N. (Proline, hydroxyproline, and one-half tryptophan N. Difference between III and IIIa.)

C. Determination of Individual Amino Acids. Differences in the chemical nature of the various amino acids have been utilized to permit the estimation by chemical means of the quantities of individual amino acids present in a protein hydrolyzate. The principal methods in use at the present time are outlined below:

1. Glycine in a protein hydrolyzate is oxidized with ninhydrin (triketohydrindene hydrate) to yield formaldehyde.
2. Alanine is oxidized with ninhydrin to yield acetaldehyde.
3. Serine is oxidized with periodic acid to yield formaldehyde which is readily determined.
4. Threonine is oxidized with periodic acid to yield acetaldehyde.
5. Phenylalanine is first nitrated to yield 3,4-dinitrophenylalanine; this is reduced to the nitroso compound, which has a purple color in alkaline solution.
6. Tyrosine, like other 3,5 unsubstituted phenols, gives an intense red color when treated with mercury salts and nitrous acid (Millon-Nasse reaction).
7. Tryptophan gives various red and purple compounds when con-

APPROXIMATE PERCENTAGE COMPOSITION OF SELECTED ANIMAL PROTEINS

	Collagen: Cattle Hide	*Gelatins*	*Elastin: Cattle*	*Milk:* Mixed Proteins*	*Casein*	*β-Lactoglobulin*	*Egg (Hen)* Whole*	*Ovalbumin*	*Phosvitin*	*Serum Albumin**	*γ-Globulin*	*Hemoglobin: Horse*	*Fibrin and Fibrinogen*	*Bence-Jones* Protein*	*Salmine*	*Clupeine*	*Pepsin*	*Chymotrypsinogen*	*Thyroglobulin*	*Insulin*	*Growth Hormone Pituitary*	*Clostridium Botulinum Toxin*
Nitrogen	18.1	18.0	17.1	15.2	15.63	15.60	14.1	15.76	11.9	16.0	16.0	16.8	16.9	18.1	31.52	31.68	14.65	16.4	15.8	15.54	(16.0)	16.3
Sulfur		0.35	0.17	1.0	0.8	1.68	1.5	1.83	<0.1	1.8	1.06	0.58	0.9	0.89	0	0	0.94	2.07		3.33	0	
Arginine	8.6	8.8	0.9	4.2	4.1	2.9	7.0	5.9	4.8	6.0	4.8	3.7	7.8	6.0	88.4	87.0	1.0	2.8	12.7	3.1	9.1	4.6
Histidine	0.7	0.7	<0.1	2.8	3.1	1.7	2.4	2.6	4.8	4.0	2.5	8.7	2.6	1.2	0	0	0.9	1.2	2.2	4.9	2.7	1.0
Lysine	5.0	5.1	0.5	8.7	8.2	11.3	7.5	6.5	5.9	12.7	8.1	8.5	9.2	7.0	0	0	1.6	7.7	3.4	2.5	7.1	7.7
Tyrosine	1.0	0.7	1.6	6.0	6.3	3.7	4.5	3.7	0.1	5.5	6.8	3.0	5.5	7.5	0	0	8.5	2.7	3.1	13.0	5.2	13.5
Tryptophan	0	0	0	1.5	1.2	1.9	1.7	1.2	0.6	1.0	2.9	1.7	3.3	1.4	0	0	2.4	5.6	2.1	0	0.9	1.9
Phenylalanine	2.4	2.1	5.0	5.5	5.0	4.8	6.3	7.7	0.6	7.0	4.6	7.7	5.8		0	0	6.4	3.8	6.7	8.1	7.9	1.2
Cystine	0.1	0.1	0.6	1.0	0.3	3.5	2.3	2.8	0.0	6.5	3.1	1.0	2.7	3.2	0	0	2.1	4.6	4.4	12.5		0.8
Methionine	0.9	0.9	0.3	3.2	3.4	3.2	4.0	5.3	0.3	1.3	1.1	1.0	2.6	0.0	0	0	1.7	1.2	1.3	0	3.0	1.1
Threonine	2.2	2.2	1.3	4.7	4.9	5.5	5.0	4.0	1.4	7.1	8.4	4.4	7.1		0	1.9	9.6	10.7		2.1		8.5
Serine	3.4	3.8	0.8	4.3	6.3	4.4	7.5	8.2	33	7.0	11.4	5.8	7.0		7.0	3.4	12.2	10.9	10.8	5.2		4.4

Leucine	3.2	3.2	8.7	11.0	9.2	15.2	9.2	9.9	1.0	12.0	9.3	15.2	7.1		0	0	10.4	8.9	12.8	13.2	12.1	10.3
Isoleucine	2.1	1.9	4.0	7.5	6.1	7.3	7.7	7.0	0.5	3.0	2.7	0?	4.8		1.4	1.0	10.8	4.9		2.8	4.0	11.9
Valine	2.7	3.1	17.4	7.0	7.2	6.2	7.8	8.8	1.1	6.0	9.7	9.0	4.1		4.1	3.6	7.1	10.3	1.5	7.8	3.9	5.3
Glutamic acid	10.8	11.7	2.1	21.5	22.4	21.5	12.6	16.5	3.4	17.0	11.8	8.2	14.5	8.6	0	0	11.9	7.4		18.6	13.0	15.6
Aspartic acid	7.5	6.3	0.6		7.1	11.4	5.7	9.3	4.4	10.3	8.8	10.6	13.0	4.7	0	0	16.0	10.9		6.8	9.0	20.1
Glycine	29.3	30.5	29.9	2.3	2.7	1.5	3.7	3.1	1.6	2.0	4.2	5.6	5.6		3.3	0	6.4	6.3	3.7	4.3	3.8	1.4
Alanine	10.0	9.2	18.9		3.0	7.1		6.7	1.5	6.2		7.4	3.7		1.5	4.7		5.8	7.4	4.5		3.9
Proline	16.5	16.3	17.0		11.3	5.0		3.8	1.0	5.1	8.1	8.5	5.7	6.9	6–8	8	5.0	3.8		2.5		2.6
Hydroxyproline	14.0	14.5			0	0?		0?		0	0?	0?	0?	0	0	0				0?		

* Calculated in g. of amino acid per 16.0 g. of nitrogen.

densed with aromatic or aliphatic aldehydes in the presence of concentrated hydrochloric or sulfuric acid (Hopkins-Cole, Voisenet-Rhode reactions).

8. Cystine is reduced to cysteine. The latter is capable of reducing phospho-18-tungstic acid to yield the deep-blue lower oxides of tungsten.

9. Methionine is treated with sodium nitroprusside to give a colored complex.

10. Aspartic acid is first precipitated as the calcium salt and then is isolated as copper aspartate.

11. Glutamic acid is oxidized with chloramine-T to β-cyanopropionic acid. The latter is hydrolyzed to succinic acid which is then determined.

12. Histidine is precipitated as the silver salt at pH 7.4 and then isolated as histidine nitranilate. Nitranilic acid is 2,5-dihydroxy-3,6-dinitro-*p*-benzoquinone.

13. Arginine is first precipitated by silver nitrate and baryta at strongly alkaline reaction and then isolated as pure arginine 2,4-dinitro-1-naphthol-7-sulfonate (arginine monoflavianate).

14. Lysine can be determined in the intact protein by virtue of the fact that almost all of the free amino groups in a protein are the ϵ-amino groups of lysine. Lysine is isolated from a protein hydrolyzate previously freed of histidine and arginine, by precipitation with phospho-24-tungstic acid and subsequent isolation as lysine picrate.

15. Hydroxylysine is precipitated with phosphotungstic acid. On treatment with periodic acid in alkaline solution, one molecule of hydroxylysine yields one molecule of formaldehyde and one of ammonia.

D. The Isotope Dilution Method of Ussing and of Schoenheimer and Rittenberg. A compound which has an abnormal isotope content is inseparable from its normal analogue by the usual laboratory procedures. Thus if an amino acid containing a known excess of an isotope is added to a protein hydrolyzate, the quantity of the amino acid present in the hydrolyzate can be calculated by a determination of the isotope content of the isolated amino acid. The yield of amino acid actually isolated is of no importance in this method.

Isotopic-Carrier Method. A mixture of amino acids is treated with a reagent containing stable or radioactive isotopes to form quantitatively a stable derivative of the desired constituent. Then an overwhelming excess, W, of the unlabeled derivative (the carrier) is added. The carrier is isolated and purified to constant isotope concentration C_c.

If C_r is the isotopic concentration of the pure isotopic derivative prepared with the same reagent, the quantity of derivative present is $W \times C_c/C_r$. Thus, one isotopic reagent suffices for the analysis of many compounds.

E. Microbiological Procedures. Various microorganisms such as *Lactobacillus arabinosus*, etc., require certain amino acids for normal growth (see p. 1062). Preparation of synthetic media lacking in only one of these amino acids permits the determination of the specific amino acid in the unknown solution. Growth of the microorganism on the synthetic medium supplemented by the solution undergoing analysis is compared with that obtained in the presence of known amounts of added amino acid. In other cases, mutants of the mold *Neurospora crassa* have been developed which require only one amino acid for growth. A number of enzymes have been separated from plant and animal sources, each of which decomposes only a single amino acid in a mixture. Thus arginine may be determined by the action of arginase, which liberates ammonia; tyrosine, histidine, lysine, and glutamic acid are each decarboxylated by specific enzymes. Quantitative determination of the carbon dioxide thus liberated is a measure of the amino acid present.

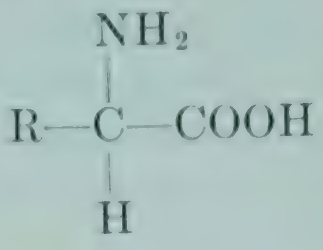
F. Chromatographic and Ion-Exchange Methods. The powerful analytical tools of paper chromatography and ion-exchange chromatography have not only been applied to the qualitative and quantitative determination of all the amino acids which commonly occur in protein hydrolyzates but have enabled investigators to find hitherto unsuspected amino acids, e.g., monoiodotyrosine, β-hydroxyphenyl-triiodotyrosine, diaminopimelic acid, etc. Plate I shows the separation of the amino acids in a protein (keratin from wool fiber) hydrolyzate on a two-dimensional paper chromatogram after staining with ninhydrin. This type of chromatogram is excellent for the qualitative identification of amino acids and it may be employed, under proper conditions, for their quantitative estimation. The quantitative or, more usually, semiquantitative estimation is based on the observation that the maximum color density of each spot is proportional to the concentration of material at that spot.

The amino acids may also be separated one from another by first adsorbing the mixture on a column of a polystyrene cation-exchange resin. The individual amino acids are then eluted from the resin with buffers of progressively increasing pH. Many small fractions of the eluate are collected separately and each fraction is analyzed for its amino acid content by heating with ninhydrin. From the data thus obtained, the quantity and identity of each amino acid in the mixture may be computed.

G. Electrolytic Separations. The α-amino acids may be divided into three groups—neutral, acidic, basic—by virtue of their charge in solutions of definite hydrogen-ion concentrations. Electrolytic separations are employed preliminary to paper chromatography.

General Properties of Amino Acids. The amino acids derived from proteins are all α-amino acids. The α carbon atom in all the acids, with the exception of glycine, is asymmetric, so that these acids are all optically active. They are white crystalline substances, the crystal form being characteristic for each acid. They are all soluble in water, except cystine and tyrosine, the latter being more soluble in hot than in cold water. With the exception of proline, they are all insoluble in alcohol, and all are insoluble in ether. They are, as a rule, all soluble in solutions of strong acids and bases. They are not precipitated by ammonium sulfate or sodium chloride, but with the exception of proline are precipitated by alcohol. They form crystalline salts with metallic bases and with mineral acids. Many of the amino acids (such as glycine, alanine, serine, and proline) have a sweet taste; some, like tryptophan and leucine, are tasteless; others, like arginine, are bitter. The amino acids are all amphoteric, ionizing both as acids and as bases by virtue of their carboxyl and amino groups, thus forming salts with alkalies and with acids.

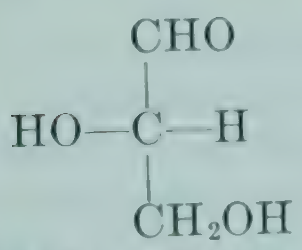
Spatial Configuration and Optical Activity of Amino Acids. The amino acids have the following general structure:



$$\begin{array}{c} NH_2 \\ | \\ R-C-COOH \\ | \\ H \end{array}$$

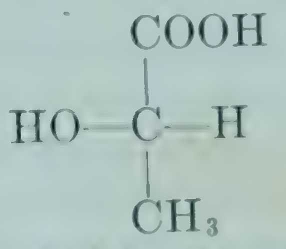
In this structure the α carbon atom is asymmetric—i.e., there are four different substituent groups. Thus all the amino acids except glycine (where $R = H$) are optically active, and are capable of existing in two different spatial forms, which are mirror images of one another, with equal and opposite optical rotatory power, and which differ solely in the arrangement of the substituent groups in space around the α carbon atom. These two different spatial arrangements are known respectively as the D and L configurations.

The configuration of a particular amino acid is based on its spatial relationship to an arbitrary reference compound, just as the configuration of D-glucose is based on that of dextrorotatory D-glyceraldehyde (see Chapter 2). This relationship, for the amino acids, is illustrated as follows:



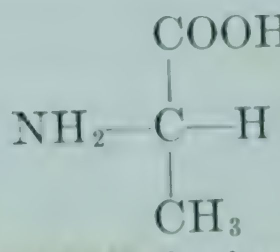
$$\begin{array}{c} CHO \\ | \\ HO-C-H \\ | \\ CH_2OH \end{array}$$

L(−)-glyceraldehyde
(levorotatory)



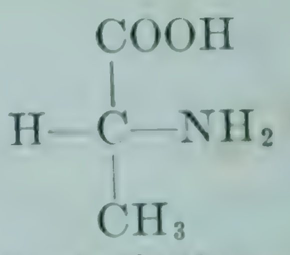
$$\begin{array}{c} COOH \\ | \\ HO-C-H \\ | \\ CH_3 \end{array}$$

L(+)-lactic acid
(dextrorotatory)



$$\begin{array}{c} COOH \\ | \\ NH_2-C-H \\ | \\ CH_3 \end{array}$$

L(+)-alanine
(dextrorotatory)



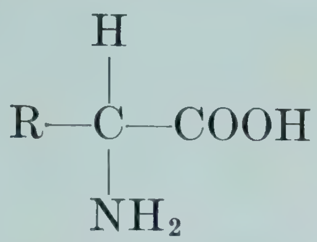
$$\begin{array}{c} COOH \\ | \\ H-C-NH_2 \\ | \\ CH_3 \end{array}$$

D(−)-alanine
(levorotatory)

Thus the naturally occurring amino acid alanine, which is dextrorotatory, may by suitable means be related structurally to either dextrorotatory lactic acid or levorotatory glyceraldehyde. Both of these have the same configuration, which in this case is known as the L configuration when referring to amino acids. As with the sugars, there is no necessary agreement between configuration and the direction of optical rotation; to indicate the latter, the signs (+) for dextrorotatory and (−) for levorotatory are used. Naturally occurring alanine is therefore L(+)-alanine; its mirror image is D(−)-alanine.

The spatial configuration of an amino acid appears to have important physiological significance. For instance, certain (but not all) of the amino acids cannot be utilized by the animal body if the configuration is opposite to that found naturally. It is an interesting fact that the bulk of the amino acids found in nature, either free or as part of proteins, have the L configuration. Certain D amino acids have however been isolated from natural sources: thus D glutamic acid is a major constituent of the capsule of *Bacillus anthracis* and related microorganisms; the naturally occurring antibiotic polypeptide, gramicidin, contains a high proportion of D amino acids; and D valine is a decomposition product of the penicillins. The significance of these stereochemical findings remains to be elucidated.

Chemical Reactions of Amino Acids. The amino acids give three general types of chemical reactions: (1) Reactions due to the presence in the molecule of the carboxyl (COOH) group, (2) reactions due to the amino (NH_2) group, and (3) reactions due to the radical R. Of these three types of reactions, those due to the carboxyl and amino groups are perfectly general reactions and are given by all the amino acids.

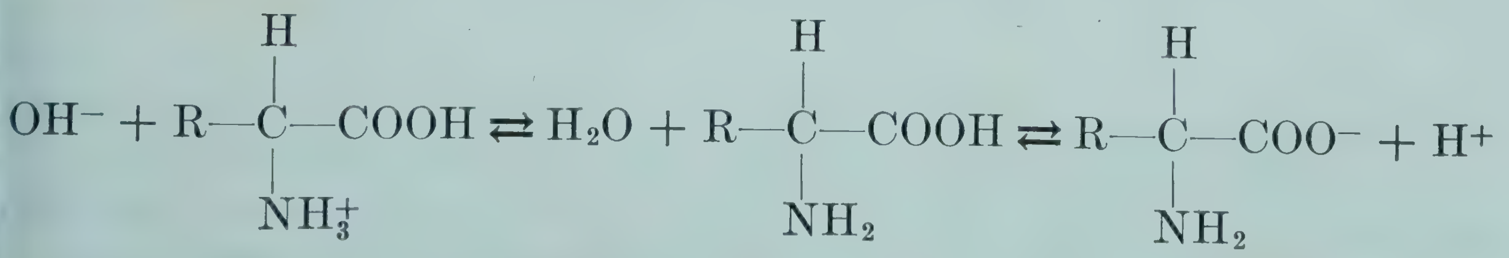


$$\mathrm{R-\underset{NH_2}{\overset{H}{C}}-COOH}$$

The reactions given by the radical R are usually specific reactions, given only by those acids which contain certain particular groups. Thus cystine gives a reaction for sulfur, tryptophan gives certain color reactions because its molecule contains the indole group, tyrosine gives other color reactions because of its phenolic group, etc. Some of these color reactions that are specific for individual amino acids are used as general color reactions for proteins since most proteins contain one or more of these acids in their molecules. The most important of these reactions are therefore described in the next chapter, under the general reactions of proteins.

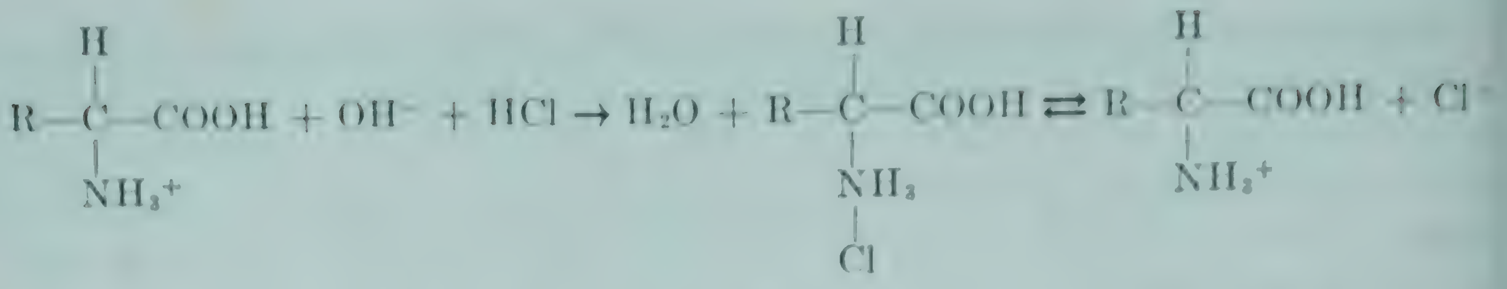
The reactions given by all amino acids by virtue of their carboxyl and amino groups are of great importance. Some of these reactions, such as the formation of a great variety of crystalline derivatives with organic and inorganic compounds, are useful in the isolation, purification, and quantitative determination of individual amino acids. Among such derivatives we may mention the ethyl esters, picrates, flavianates, picrolonates, copper, barium, cadmium, calcium, nickel, and zinc salts, and double salts with compounds such as phosphotungstic acid, chloroplatinic acid, gold chloride, silver nitrate, mercuric acetate, and mercuric chloride. Many of these reactions are given by the intact protein, since the latter always contains a certain number of free amino and carboxyl groups. The more important of these reactions of amino acids which give us an insight into the complex chemical and physicochemical behavior of the protein molecule are discussed below.

REACTIONS OF AMINO ACIDS WITH ACIDS AND BASES. Since the amino acids contain both carboxyl and amino groups, they behave like typical amphoteric compounds, ionizing both as acids and as bases:



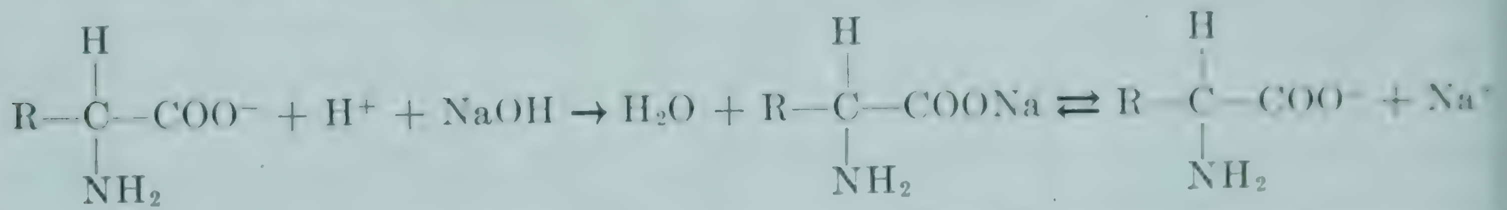
$$\mathrm{OH^- + R-\underset{NH_3^+}{\overset{H}{C}}-COOH \rightleftarrows H_2O + R-\underset{NH_2}{\overset{H}{C}}-COOH \rightleftarrows R-\underset{NH_2}{\overset{H}{C}}-COO^- + H^+}$$

The addition of acid favors the ionization to the left and leads to the formation of salts of the amino acid similar to those formed by ammonia and other substituted ammonia derivatives. With hydrochloric acid, for example, a hydrochloride is formed which ionizes into a positive amino acid ion and a negative chlorine ion:



$$R-\overset{\overset{H}{|}}{\underset{\underset{NH_3^+}{|}}{C}}-COOH + OH^- + HCl \rightarrow H_2O + R-\overset{\overset{H}{|}}{\underset{\underset{\underset{\underset{Cl}{|}}{NH_3}}{|}}{C}}-COOH \rightleftarrows R-\overset{\overset{H}{|}}{\underset{\underset{NH_3^+}{|}}{C}}-COOH + Cl^-$$

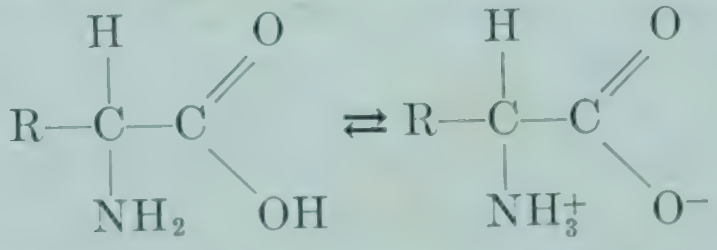
With alkalies, such as sodium hydroxide, the ionization proceeds to the right and leads to the formation of a sodium salt of the amino acid, which is ionized. This gives a positive sodium ion and a negative amino acid ion:



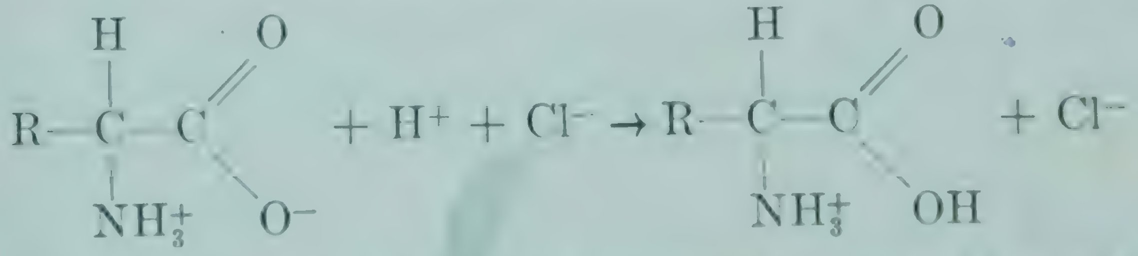
$$R-\overset{\overset{H}{|}}{\underset{\underset{NH_2}{|}}{C}}-COO^- + H^+ + NaOH \rightarrow H_2O + R-\overset{\overset{H}{|}}{\underset{\underset{NH_2}{|}}{C}}-COONa \rightleftarrows R-\overset{\overset{H}{|}}{\underset{\underset{NH_2}{|}}{C}}-COO^- + Na^+$$

In acid solutions, therefore, the amino acid carries a positive charge and in an electrical field migrates to the cathode, but in alkaline solutions it carries a negative charge and migrates to the anode. For every amino acid there is a definite hydrogen-ion concentration, specific for that acid, at which the degrees of acid and basic ionization are equal. At this particular pH, known as the isoelectric point, the amino acid is electrically neutral and will not migrate in an electrical field.

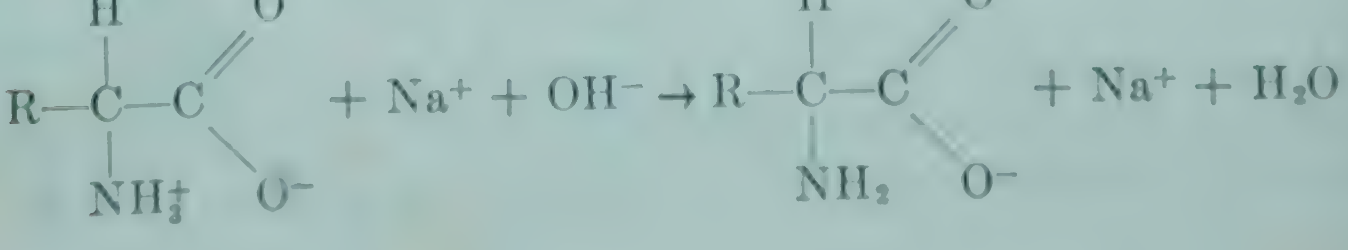
In past years there has been a great accumulation of experimental evidence indicating that this electrically neutral form of the amino acid consists of a mixture of undissociated molecules and a tautomeric form, known as a *zwitterion*, in which both the amino and carboxyl groups



are ionized to the same extent. In solutions of aliphatic amino acids, more than 99 per cent of the molecules are present in the form of zwitterions; in the aromatic acids the two forms are present in approximately equal amounts. The addition of acid to the zwitterion suppresses the ionization



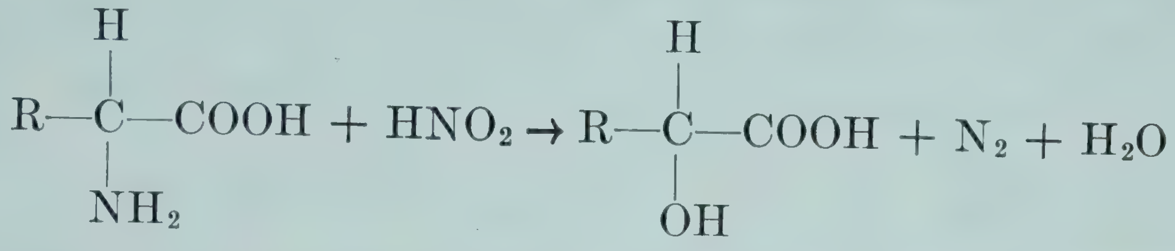
of the carboxyl group, thus leaving a positively charged ion which is free to form salts with the acid. Similarly, alkali suppresses the ionization of the amino group, thus leaving a negatively charged ion which can form salts with the base. Thus the effects of adding acid or alkali to the amino



acid are the same regardless of whether the neutral amino acid is considered to be an undissociated molecule or a zwitterion.

These reactions of amino acids with acids and with bases are of great importance in protein chemistry since, as we shall see in Chapter 5, a great many of the physicochemical reactions of proteins are explainable on the basis that the protein molecule contains a definite number of free amino and free carboxyl groups, the exact number of each being characteristic of each particular protein. Depending upon the pH of the solution, therefore, proteins combine with acids and bases, and carry a preponderance of either positive or negative charges, or behave as though they were electrically neutral.

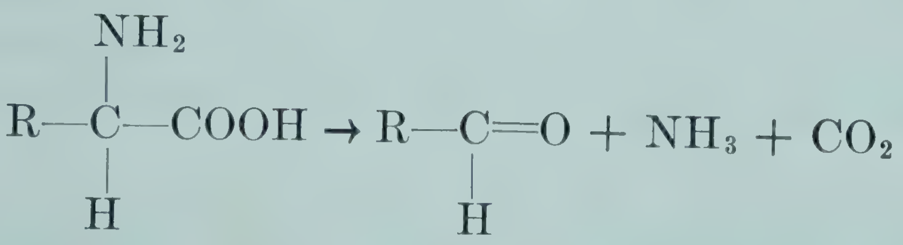
REACTIONS OF AMINO ACIDS WITH NITROUS ACID. The amino acids, as their general formula indicates, are primary amines, and like all such amines yield nitrogen when treated with nitrous acid. This reaction



$$R\text{—}\underset{NH_2}{\overset{H}{C}}\text{—}COOH + HNO_2 \rightarrow R\text{—}\underset{OH}{\overset{H}{C}}\text{—}COOH + N_2 + H_2O$$

forms the basis for Van Slyke's method for the determination of free amino groups, as has already been indicated. For this purpose use is made of a specially devised apparatus in which the nitrogen gas evolved during the reaction is collected and its volume measured. This reaction is important for the determination of free amino groups in amino acids, or mixtures of amino acids, and is also used in estimating the amounts of amino acids in biological fluids, such as blood. It is also used to determine the percentage of the total nitrogen of the protein that is present in the form of free amino nitrogen. Since the latter increases during acid or enzymatic hydrolysis, the method is also of great value in determining the rate and extent of protein degradation by any of the hydrolytic agents.

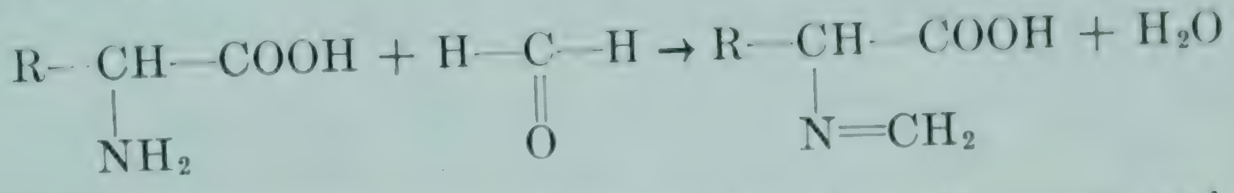
REACTIONS OF AMINO ACIDS WITH NINHYDRIN. The amino acids in general react with the compound ninhydrin ($C_6H_4{\cdot}(CO)_3{\cdot}H_2O$, triketohydrindene hydrate) to yield carbon dioxide, ammonia, and usually (but not always) an aldehyde containing one fewer carbon atom than the original amino acid.



$$R\text{—}\underset{H}{\overset{NH_2}{C}}\text{—}COOH \rightarrow R\text{—}\underset{H}{C}{=}O + NH_3 + CO_2$$

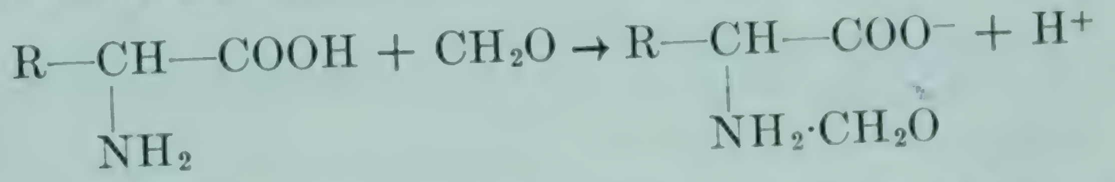
This reaction has been made the basis for several different types of quantitative methods for the determination of amino acids. These are based upon (1) the color change which results from the reaction (see pp. 18 and 172), (2) determination of the ammonia produced, and (3) measurement by gasometric means of the carbon dioxide evolved. This latter procedure has been accurately established by Van Slyke and his associates as possibly the most satisfactory method available for the determination of α-amino nitrogen (in terms of CO_2), exceeding the nitrous acid method in specificity for this purpose. For a description of this method as applied to the determination of amino acid nitrogen in urine, see p. 892.

REACTIONS OF AMINO ACIDS WITH FORMALDEHYDE. The carboxyl group of the simple amino acids is not readily titratable with alkali under ordinary conditions, presumably because of the influence of the neighboring amino group. In 1899 Schiff observed that in the presence of formaldehyde the amino acids became as readily titratable as any simple organic acid. Sørensen formulated the reaction between amino acids and formaldehyde as follows:



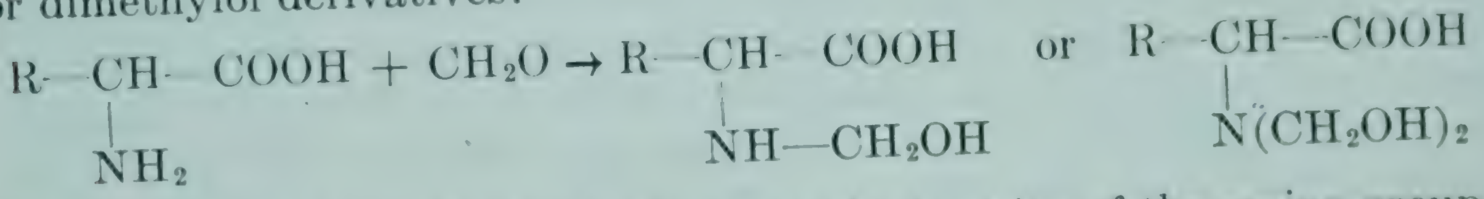
$$R{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH + H{-}\underset{\displaystyle O}{\underset{\|}{C}}{-}H \rightarrow R{-}\underset{\displaystyle N{=}CH_2}{\underset{|}{CH}}{-}COOH + H_2O$$

There is considerable evidence against this view, however, and Harris believes that only an amino acid-formaldehyde complex is formed:



$$R{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH + CH_2O \rightarrow R{-}\underset{\displaystyle NH_2{\cdot}CH_2O}{\underset{|}{CH}}{-}COO^- + H^+$$

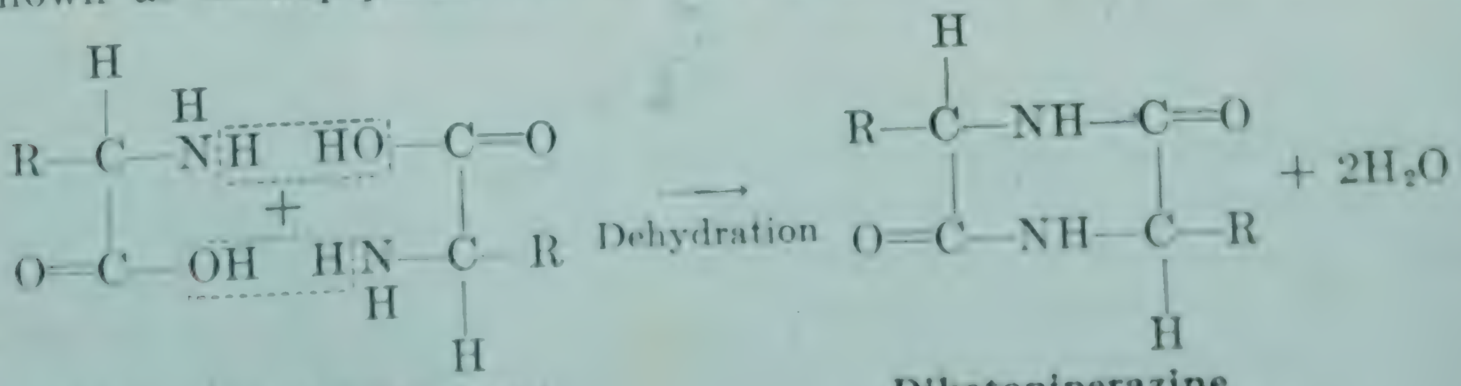
Others have suggested that HCHO reacts with amino acids to give mono- or dimethylol derivatives:



$$R{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH + CH_2O \rightarrow R{-}\underset{\displaystyle NH{-}CH_2OH}{\underset{|}{CH}}{-}COOH \quad \text{or} \quad R{-}\underset{\displaystyle N(CH_2OH)_2}{\underset{|}{CH}}{-}COOH$$

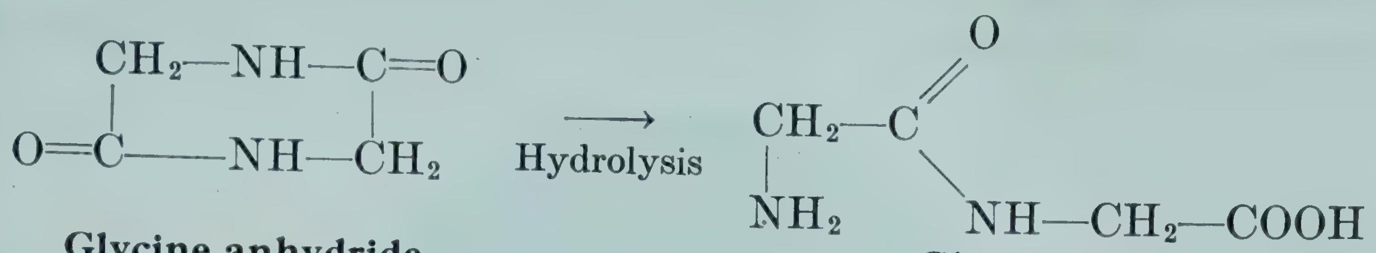
The presence of formaldehyde decreases the basicity of the amino group, permitting the carboxyl group to exert its maximum acidity. This acidity may then be titrated with standard sodium hydroxide, using phenolphthalein as indicator. This reaction forms the basis for Sørensen's *formol titration* method for the estimation of free carboxyl groups in amino acids and mixtures of amino acids. It is also widely used for determining the increase in carboxyl groups which accompanies the enzymatic hydrolysis of proteins.

REACTIONS OF AMINO ACIDS WITH AMINO ACIDS. Many of the amino acids readily form anhydrides when their solutions are evaporated. This reaction involves a condensation between the amino group of each molecule with the carboxyl group of the other, the resulting compounds being known as *diketopiperazines*. In 1901 Fischer and Fourneau subjected



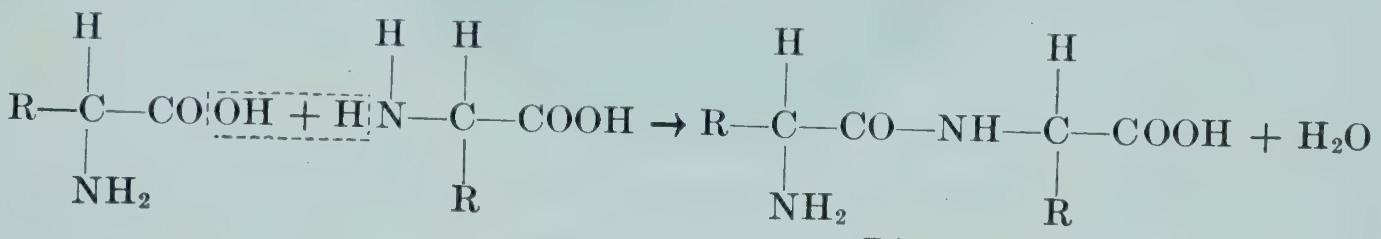
2 Molecules of amino acid **Diketopiperazine**

glycine anhydride to weak hydrolysis with acids and obtained a compound, glycyl-glycine, in which the amino group of one acid was combined with the carboxyl group of the other. This was the starting point of a great



Glycine anhydride **Glycyl-glycine**

many researches whereby Fischer and his co-workers, using various derivatives of amino acids, prepared a large number of similar compounds between amino acids, called *dipeptides*. Although the details of these methods would be out of place in a book of this character, the reactions involved were chosen so that the amino group of one acid always combined with the carboxyl group of the other, resulting in the so-called *peptide linkage*. Since these dipeptides still contained a free amino and a carboxyl group, by the use of various ingenious methods Fischer was able to lengthen these chains of amino acids, forming polypeptides containing as many as 18 acids. According to the modern conceptions of protein structure, the protein molecule consists in large part, at least, of amino acids linked together through their amino and carboxyl groups. The evidence for this point of view will be discussed more fully in the section on the structure of the protein molecule in Chapter 5.

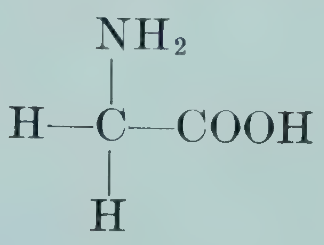


$$R-\underset{NH_2}{\overset{H}{C}}-CO\boxed{OH + H}\overset{H}{N}-\underset{R}{\overset{H}{C}}-COOH \rightarrow R-\underset{NH_2}{\overset{H}{C}}-CO-NH-\underset{R}{\overset{H}{C}}-COOH + H_2O$$

Dipeptide

DISCUSSION OF THE INDIVIDUAL AMINO ACIDS

Glycine, $C_2H_5O_2N$ (***aminoacetic acid, glycocoll***).



$$H-\underset{H}{\overset{NH_2}{C}}-COOH$$

Glycine was the first amino acid to be isolated as a primary decomposition product of the proteins. Glycine is prepared synthetically by the action of ammonia on monochloroacetic acid. It crystallizes in rather large colorless monoclinic crystals usually in four-sided prisms. Glycine possesses no asymmetric carbon atom and is therefore not optically active.

In addition to being present in many animal proteins, glycine is found in the body as a constituent of various nonprotein nitrogenous compounds. It is a component of (1) glutathione, a tripeptide of glutamic acid, cysteine, and glycine (see p. 146); (2) glycocholic acid, a compound of glycine and cholic acid, found in the bile (see p. 410); and (3) hippuric acid, or benzoyl-glycine, which is found in the urine after the ingestion of benzoic acid or compounds which give rise to benzoic acid in metabolism. The reaction leading to the formation of hippuric acid from benzoic acid and glycine is as follows:

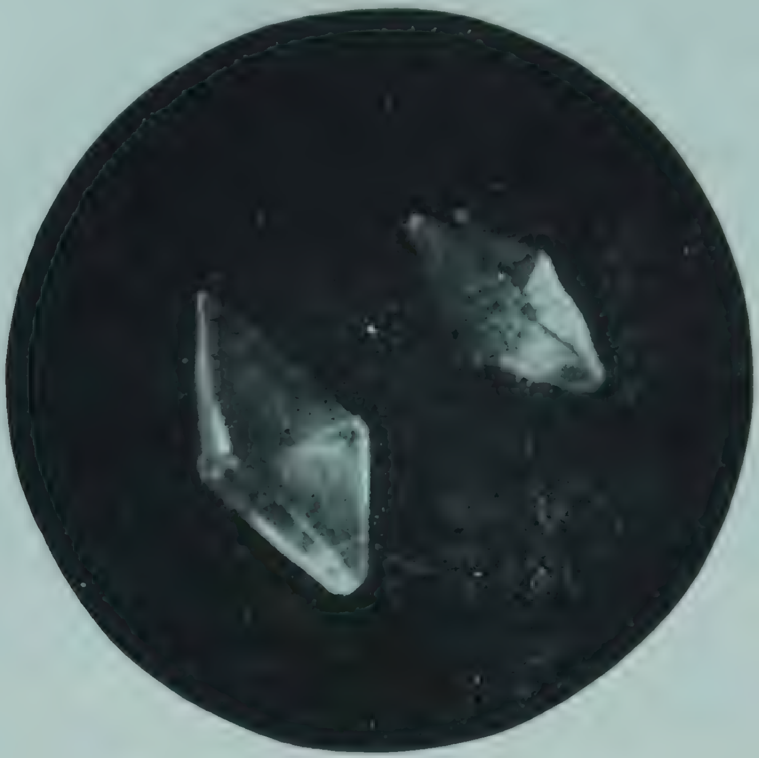


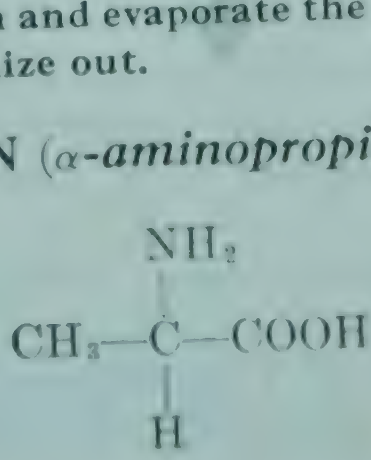
FIG. 38. GLYCINE (SYNTHETIC).
See also Fig. 103.
From Keenan: *J. Biol. Chem.*, **62**, 163, (1924).

$$\underset{\textbf{Benzoic acid}}{C_6H_5COOH} + \underset{\textbf{Glycine}}{H_2N{-}CH_2COOH} \rightarrow \underset{\textbf{Hippuric acid}}{C_6H_5CO{-}NH{-}CH_2COOH} + H_2O$$

EXPERIMENTS ON GLYCINE

1. *Preparation of Glycine from Hippuric Acid.* Put 5 g. of hippuric acid in a 100-ml. round-bottom flask. Attach the flask to a reflux condenser after adding 20 to 25 ml. of 30 per cent sulfuric acid. Boil the contents of the flask on a wire gauze for one hour, cool under the tap, and filter. The boiling acid splits the hippuric acid into benzoic acid and glycine. The benzoic acid, being quite insoluble in cold acid, begins to crystallize out and is retained on the paper. In order to remove the remainder of the benzoic acid from the filtrate, shake the filtrate with twice its volume of ether in a separatory funnel. The filtrate is next diluted with three volumes of water and heated with sufficient barium carbonate, in the presence of caprylic alcohol to minimize foaming, until the sulfuric acid has been precipitated out as barium sulfate. Remove the barium sulfate by centrifugation and evaporate the filtrate on a water bath until the glycine begins to crystallize out.

L(+)-Alanine, $C_3H_7O_2N$ (*α-aminopropionic acid*).



$$\begin{array}{c} \quad\;\; NH_2 \\ \quad\;\; | \\ CH_3{-}C{-}COOH \\ \quad\;\; | \\ \quad\;\; H \end{array}$$

Alanine is best prepared from silk, in which it occurs to the extent of approximately 25 per cent. It has a sweetish taste, and is dextrorotatory.

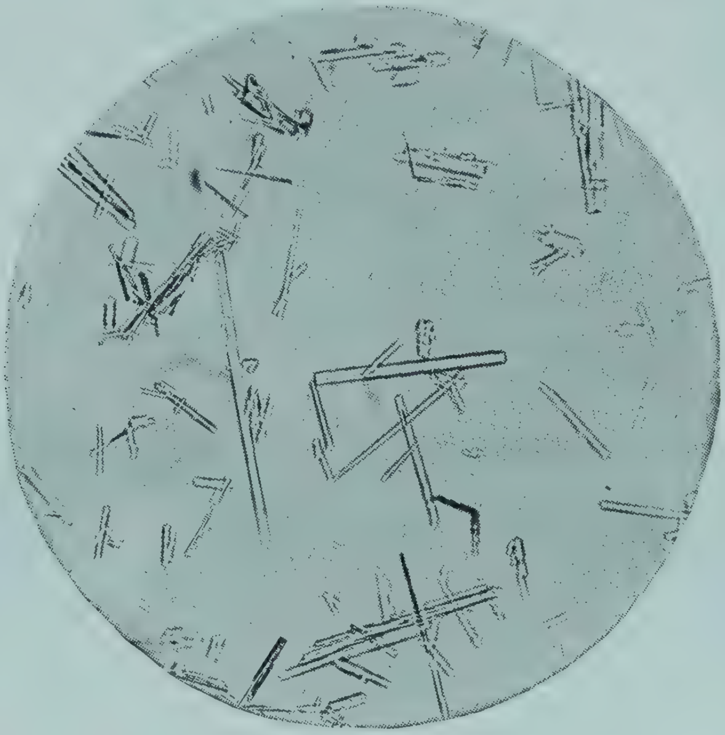
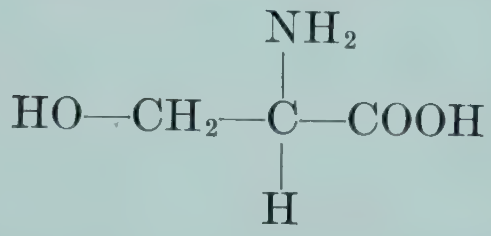


FIG. 39. ALANINE.
From Keenan: *J. Biol. Chem.*, **62**, 163 (1924).

L(—)-Serine, $C_3H_7O_3N$ (*β-hydroxy-α-aminopropionic acid*).



$$HO—CH_2—\underset{\displaystyle H}{\overset{\displaystyle NH_2}{\underset{|}{\overset{|}{C}}}}—COOH$$

Serine crystallizes from water solution as thin, irregular plates and has a sweet taste. It melts at about 245° and is soluble in 23 times its weight of water at room temperature.

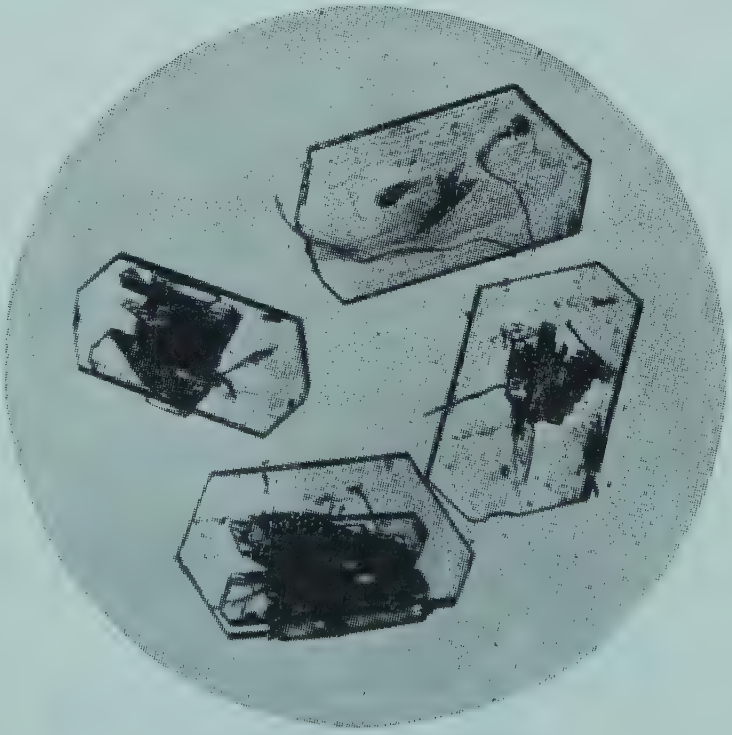
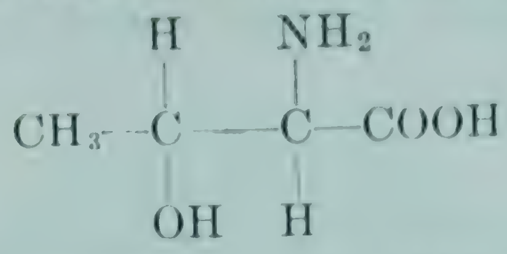


FIG. 40. SERINE.
From Keenan: *J. Biol. Chem.*, **62**, 163, (1924).

L(—)-Threonine, $C_4H_9O_3N$ (*α-amino-β-hydroxy-n-butyric acid*).



$$CH_3-\underset{OH}{\overset{H}{C}}-\underset{H}{\overset{NH_2}{C}}-COOH$$

The α carbon atom of naturally occurring threonine has the L configuration, as shown. When, however, the configuration of the molecule as a whole is considered, it is seen to resemble that of the sugar D-threose (whence the name) so that this amino acid is sometimes referred to as D(–)-threonine.

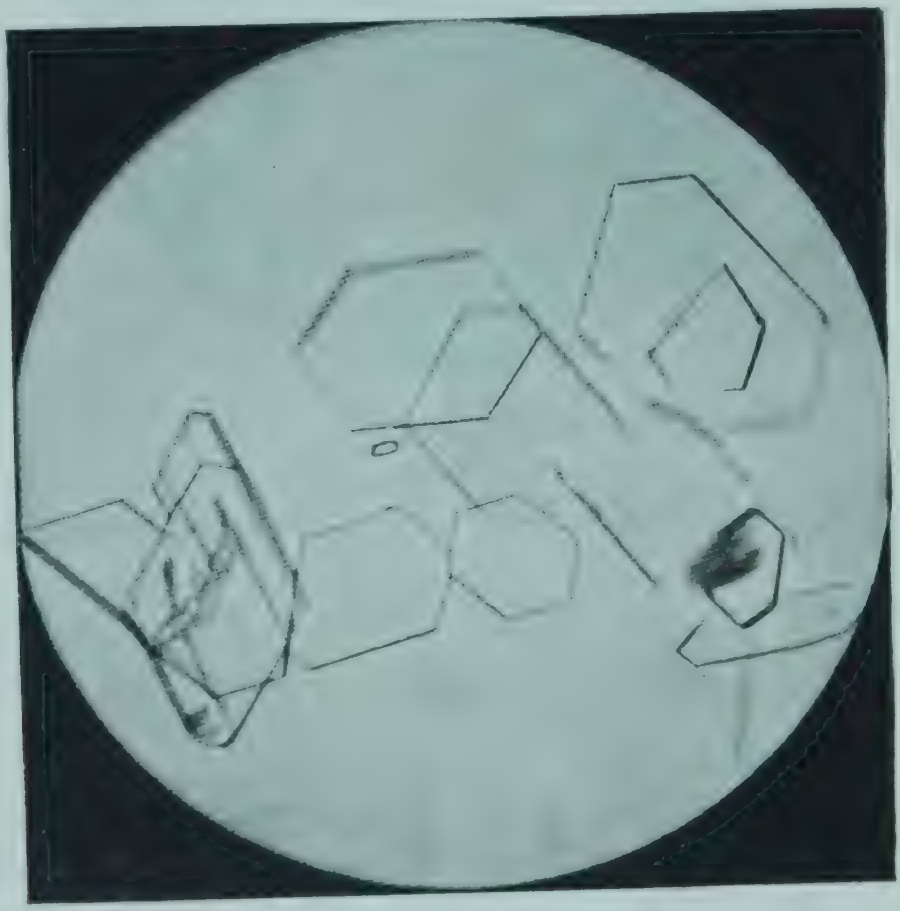
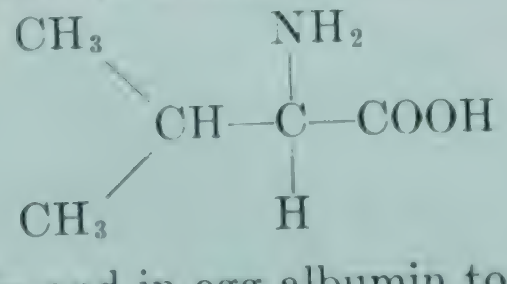


FIG. 41. THREONINE.

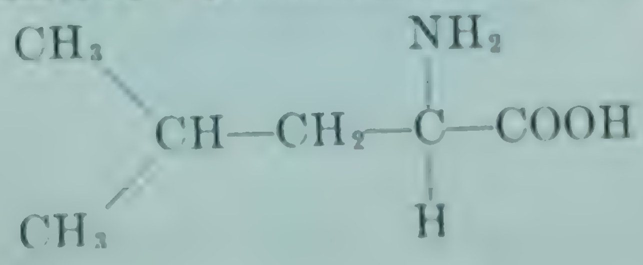
From McCoy, Meyer, and Rose: *J. Biol. Chem.*, **112**, 288 (1935).

L(+)-Valine, $C_5H_{11}O_2N$ (*α-aminoisovaleric acid*).



Valine occurs in casein and in egg albumin to the extent of 6 to 7 per cent. It is made synthetically by the interaction of ammonia and α-bromoisovaleric acid under pressure in which process the bromine is split off as HBr and the amino group takes the α-position originally occupied by the bromine.

L(—)-Leucine, $C_6H_{13}O_2N$ (*α-aminoisocaproic acid*).



Leucine is found in almost all proteins. Free leucine has been found pathologically in urine (in acute yellow atrophy of the liver, phosphorus poisoning, and acute febrile conditions). Pure leucine crystallizes in shiny, white, extremely thin plates. Inactive leucine tastes slightly sweet, D(+)-leucine tastes quite sweet, and L(−)-leucine slightly bitter.

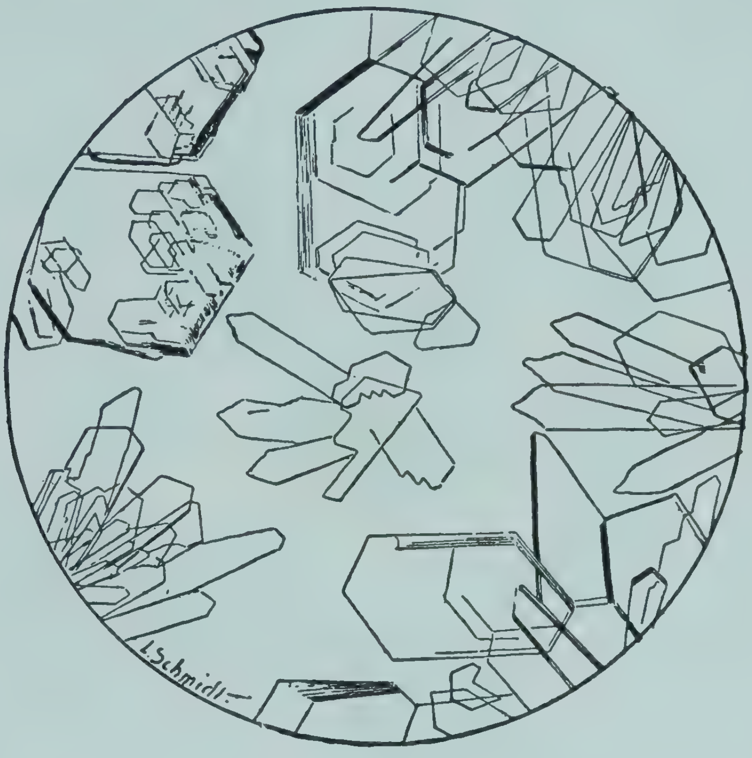


FIG. 42. LEUCINE.

For the material from which the above crystals were prepared, as well as those reproduced in Figs. 43 to 45 and 47 to 48, the authors are indebted to the late Dr. Thomas B. Osborne.

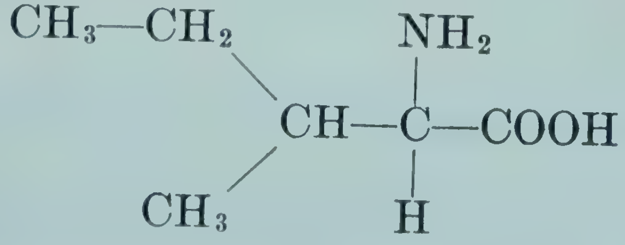
EXPERIMENTS ON LEUCINE

Preparation of Leucine. **In a 2-liter flask, place 1 liter of defibrinated blood and gradually add 150 ml. of concentrated sulfuric acid, shaking well during the additions. Boil on a sand bath for 12 to 14 hours, being careful to shake continually until it boils evenly. To the hot liquid add a solution of barium hydroxide until the mixture is alkaline to litmus. Filter on a Buchner funnel. Make the filtrate acid to litmus with dilute H_2SO_4, decolorize with 20 g. of activated charcoal, filter, and concentrate in a porcelain dish over an open flame to 500 ml. and filter again. Make the filtrate faintly alkaline to litmus through the addition of ammonia and concentrate on a boiling water bath until a crystalline mass forms on top of the liquid. Cool for 24 hours in the icebox. Filter on a Buchner funnel and press the water out of the crystals. Recrystallize from 70 per cent alcohol.**

Make the following tests upon the leucine crystals already prepared, or upon some pure leucine furnished by the instructor.

1, 2 and 3. Do these experiments according to the directions given for Tyrosine (p. 138).

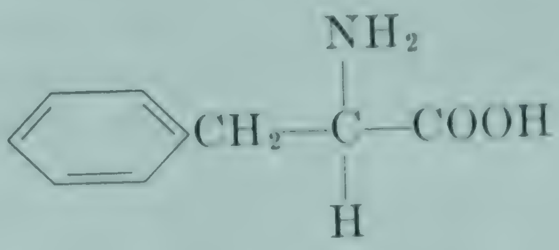
L(+)-Isoleucine, $C_6H_{13}O_2N$ (***β-methyl-α-aminovaleric acid***).



$$CH_3-CH_2-\underset{CH_3}{CH}-\overset{NH_2}{\underset{H}{C}}-COOH$$

Isoleucine has a bitter taste and it crystallizes in plates like leucine.

L(—)-Phenylalanine, $C_9H_{11}O_2N$ (*β-phenyl-α-aminopropionic acid*).



Phenylalanine is easily soluble in hot water, quite insoluble in cold water, and only slightly soluble in ethyl and methyl alcohols. It has a bitter taste.

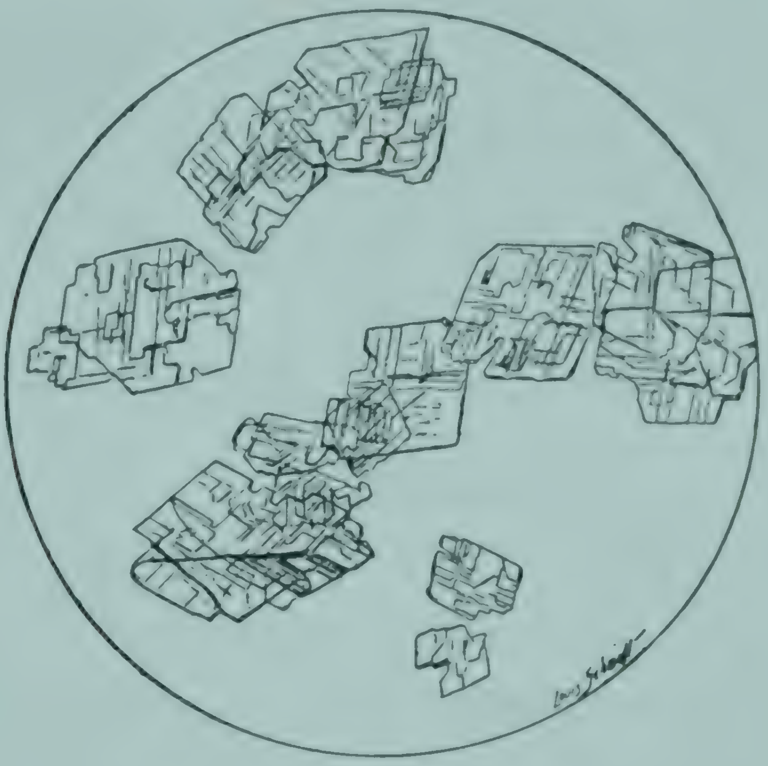
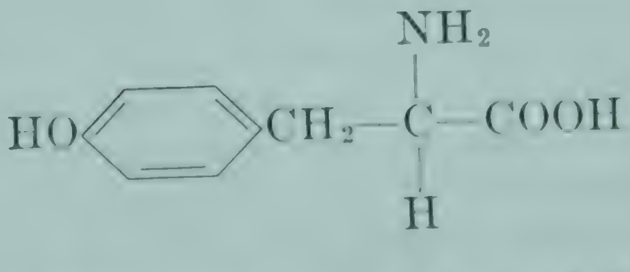


FIG. 43. PHENYLALANINE.

L(—)-Tyrosine, $C_9H_{11}O_3N$ (*β-parahydroxyphenyl-α-aminopropionic acid*).



Of all the amino acids tyrosine is the least soluble in water (1 part in 2500 of water at room temperature). It is insoluble in ether, alcohol, acetone, and glacial acetic acid, but readily soluble in dilute alkalis and acids. It crystallizes from water in sheathlike groups of fine white needles. Tyrosine is found in most proteins but is virtually absent in gelatin (0.3 per cent). It may be detected in the faintest traces by means of Millon's reaction. (See p. 169.)

EXPERIMENTS ON TYROSINE

Preparation of Tyrosine. **Introduce 200 g. of commercial casein into a 3-liter pyrex flask, add a liter of cold water, and shake well. With continuous shaking, slowly add a liter of boiling water to this mixture. Now add 40 ml. of 20 per**

cent sodium hydroxide to dissolve the casein, and adjust the reaction of the casein solution to pH 8 by the addition of normal sodium hydroxide.[4]

Preserve the casein solution and diminish oxidase action by adding 15 ml. of toluol and 2 g. of sodium fluoride dissolved in about 10 ml. of hot water. Shake well, add trypsin in the form of some commercial preparation,[5] and, after mixing the contents of the flask thoroughly, stopper the flask and place it in an incubator at 38° to 40°. Shake the flask thoroughly every day, without removing the stopper, and at the end of the fourth day add an additional quantity of trypsin in one of the forms mentioned. After permitting the digestion to continue for a second period of four days, remove the flask from the incubator, allow it to stand at room temperature for at least 24 hours, then filter off the precipitate of tyrosine, undigested casein, etc. Treat the residue with dilute sulfuric acid (5 ml. of concentrated sulfuric acid in 250 ml. of water) to dissolve the tyrosine, filter through a pleated paper,[6] add 10 ml. of concentrated ammonium hydroxide to the filtrate, and heat on a boiling water bath. The solution, which should now be acid to litmus, is carefully neutralized by the addition of ammonium hydroxide and allow to cool. Tyrosine, contaminated with more or less calcium phosphate, should crystallize out. Filter off the tyrosine by suction, suspend it in 300 ml. of water in a flask, heat to boiling, add 5 ml. of concentrated ammonium hydroxide, and boil for 15 minutes. Filter off the insoluble calcium phosphate, neutralize the tyrosine filtrate with 5 per cent H_2SO_4, and allow it to stand. Filter off the tyrosine crystals by suction, wash well with cold water and alcohol in turn, and dry in an oven or incubator. If the tyrosine crystals are not well formed (see Fig. 44) they may be recrystallized from hot water (solubility 1:154).

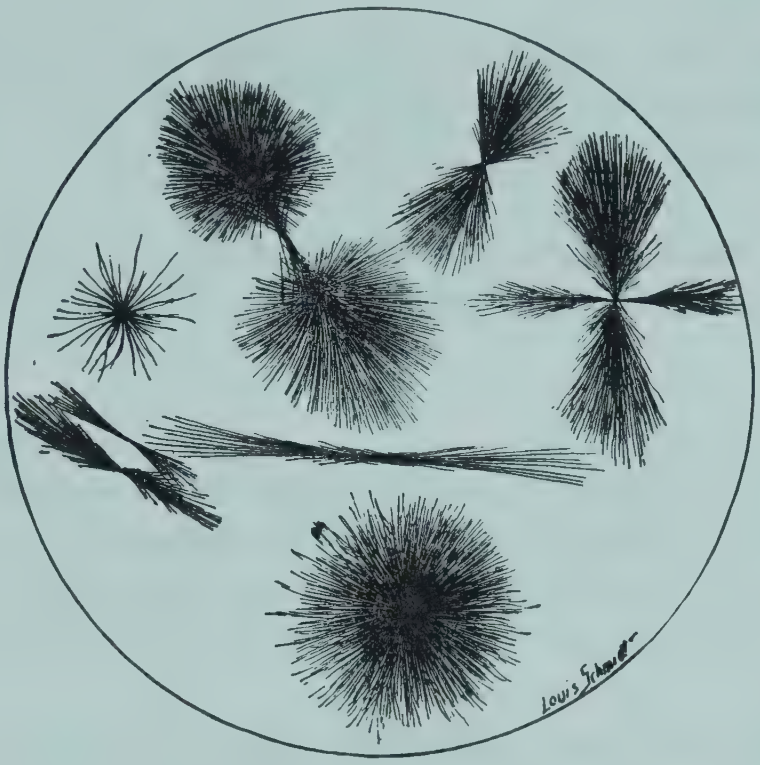


FIG. 44. TYROSINE.

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[4] To do this take 10 ml. of the casein mixture, add 10 drops of cresol red and titrate with 0.2 N NaOH (using a microburet or a 1-ml. pipet graduated in 0.01 ml.) until a reddish-purple color is obtained. Multiply this titration volume by 40 and add this quantity of normal sodium hydroxide to the casein solution in the pyrex flask. Shake the mixture at frequent intervals after the hydroxide is added. The reaction should now be acid to phenolphthalein and alkaline to cresol red.

[5] If pure trypsin is not available the enzyme may be added in the form of pancreatin.

[6] This filtration can be speeded up by the use of a diatomaceous-earth filter aid.

Make the following tests with the tyrosine crystals prepared in the above experiment, or upon some pure tyrosine furnished by the instructor.

1. *Microscopic Examination*. Place a minute crystal of tyrosine on a slide, add a drop of water, cover with a cover glass, and examine microscopically. Now run more water under the cover glass and warm in a Bunsen flame until the tyrosine has dissolved. Allow the solution to cool slowly, then examine again microscopically, and compare the crystals with those shown in Fig. 44.

2. *Solubility*. Try the solubility of very small amounts of tyrosine in cold and hot water, cold and hot 95 per cent alcohol, dilute NH_4OH, dilute KOH, and dilute HCl.

3. *Sublimation*. Place a little tyrosine in a dry test tube, heat gently, and notice that the material does not sublime. How does this compare with leucine?

4. *Hofmann's Reaction*. This is the name given to Millon's reaction when employed to detect tyrosine. Add about 3 ml. of water and a few drops (avoid an excess) of Millon's reagent to a little tyrosine in a test tube. Upon dissolving the tyrosine by heat the solution gradually darkens and may assume a dark-red color. What group does this test show to be present in tyrosine?

5. *Sulfuric Acid Test* (*Piria*). Warm a little tyrosine on a watch glass on a boiling water bath for 20 minutes with 3 to 5 drops of concentrated H_2SO_4. Tyrosine-sulfuric acid is formed in the process. Cool the solution and wash it into a small beaker with water. Now slowly add $CaCO_3$ in substance with stirring, until the reaction of the solution is no longer acid. Filter, concentrate the filtrate, and add it to a few drops (avoid an excess) of very dilute neutral ferric chloride. A purple or violet color, due to the formation of the ferric salt of tyrosine-sulfuric acid, is produced. This is one of the most satisfactory tests for the identification of tyrosine.

6. *Formaldehyde-Sulfuric Acid Test* (*Mörner*). Add about 3 ml. of Mörner's reagent[7] to a little tyrosine in a test tube, and gently raise the temperature to the boiling point. A green color results.

7. *Folin's Test*. To 1 to 2 ml. of the solution to be tested add an equal volume of "phenol reagent" (the Folin-Ciocalteu modification[8] is satisfactory) and 3 to 10 ml. of a saturated solution of sodium carbonate. A blue color is given by tyrosine. The reaction has a sensitivity of one part in one million. Tryptophan also gives this reaction.

L-Diiodotyrosine and L-Thyroxine. When tyrosine or a protein containing tyrosine is incubated at 37° with sodium bicarbonate and iodine, both diiodotyrosine and thyroxine are formed.

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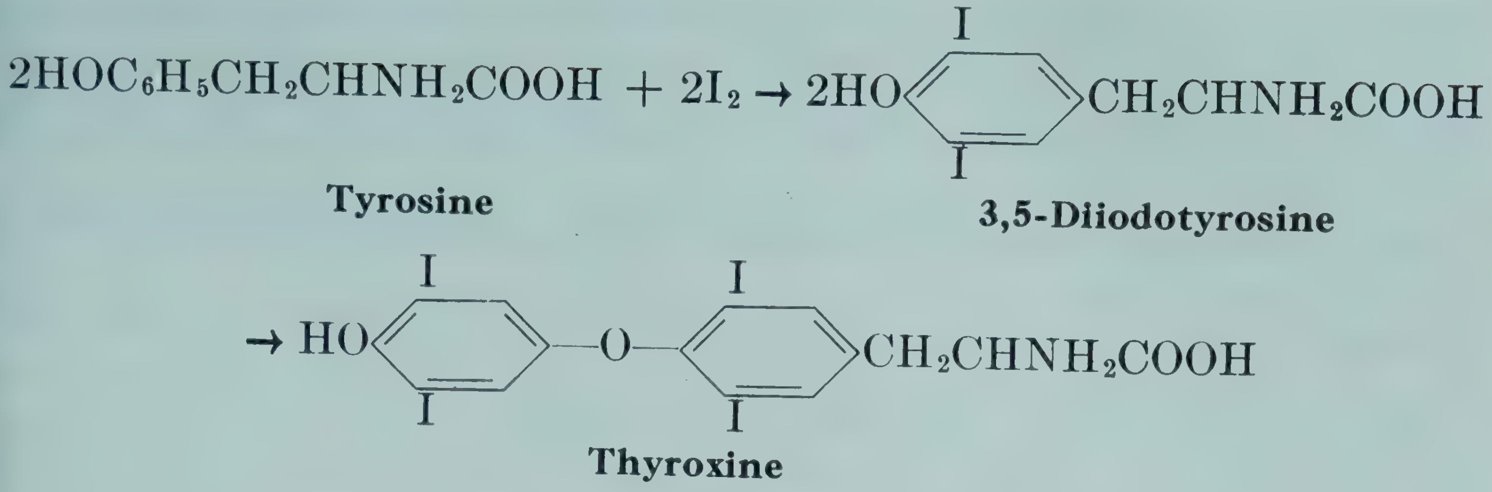
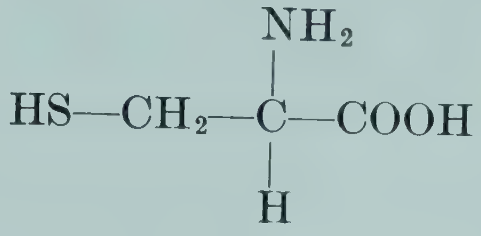
[7] Mörner's reagent is prepared by thoroughly mixing 1 ml. of formalin, 45 ml. of distilled water, and 55 ml. of concentrated sulfuric acid.

[8] See Appendix.


2HOC₆H₅CH₂CHNH₂COOH + 2I₂ → 2HO
I
I
CH₂CHNH₂COOH
Tyrosine
3,5-Diiodotyrosine
→ HO
I
I
O
I
I
CH₂CHNH₂COOH
Thyroxine

L(—)-Cysteine, $C_3H_7O_2NS$ (*β-thiol-α-aminopropionic acid*).



$$HS{-}CH_2{-}\overset{\displaystyle NH_2}{\underset{\displaystyle H}{C}}{-}COOH$$

This amino acid is recognized as being present as such in the intact protein molecule but it is not ordinarily obtained as one of the products of protein hydrolysis unless precautions are taken against oxidation, since it is readily converted by oxidation of the —SH group into various sulfinic and sulfonic acids and into cystine. The relation between cysteine and cystine may be illustrated as follows:

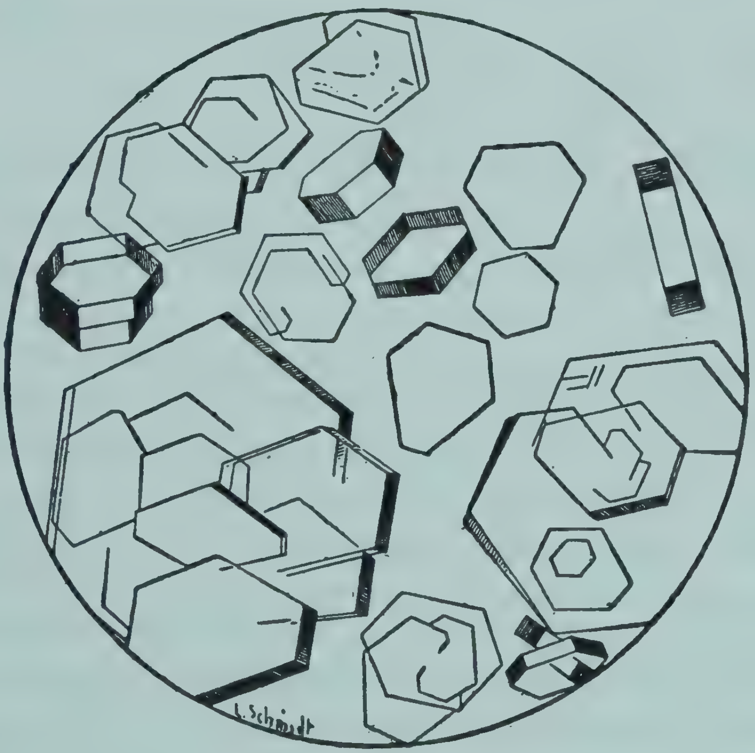
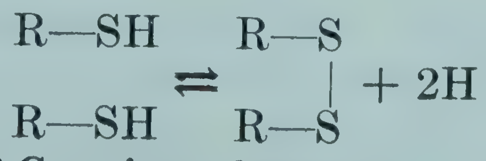


FIG. 45. CYSTINE.

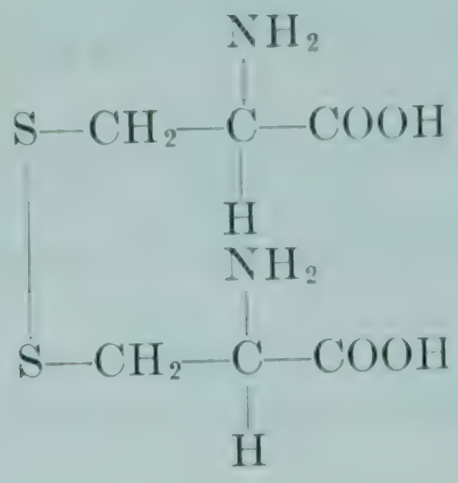


$$\begin{matrix} R{-}SH \\ R{-}SH \end{matrix} \rightleftharpoons \begin{matrix} R{-}S \\ | \\ R{-}S \end{matrix} + 2H$$

2 Cysteine **Cystine**

This reversible oxidation-reduction reaction involving the sulfhydryl group (—SH) appears to have considerable physiological significance. Thus it accounts for the oxidation and reduction reactions of glutathione, which contains cysteine, and it appears that adjacent polypeptide chains in the protein molecule are linked together through the oxidation of cysteine sulfhydryl groups. Experiments on cysteine have been grouped with those on cystine.

L(—)-Cystine, $C_6H_{12}O_4N_2S_2$ *(di(β-thiol-α-aminopropionic acid)).*



Cystine is obtained in greatest amount as a product of the hydrolysis of keratin-containing tissue such as horn, hoof, feather, and hair. It crystallizes in characteristic hexagonal plates which are only very slightly soluble in cold water and in alcohol. It dissolves readily in mineral acids or in alkalis but it is insoluble in acetic acid.

EXPERIMENTS ON CYSTINE AND CYSTEINE

***Preparation of Cysteine by the Cuprous Oxide Method** (after Lucas and Beveridge)*. **Place 300 g. of human hair (barbershop sweepings) in a 2.5-liter flask, and pour in 500 ml. of ether. Boil under a reflux condenser on a water bath for a few minutes to dissolve the oil and fat on the hair. Pour off the ether, and to the hair add 1600 ml. of 9N HCl. Boil the solution under reflux for 11 hours. Concentrate the solution in vacuo to remove excess HCl, then dissolve the residue in hot water and filter off the humin. Wash the precipitate with dilute HCl. Concentrate the filtrate and washings to a thick syrup. Take up the residue in hot water and again concentrate, this time to dryness. Repeat this process twice more. Dissolve the final residue in 1200 ml. of water.**

Grind the amino acid solution with 300 g. of red cuprous oxide in a mortar for 30 minutes. Then slowly add the suspension with stirring to 12 liters of ice water. Cool at 4° C. for 2 hours. Filter the grayish-white cysteine copper mercaptide and wash the precipitate with ice water.

Suspend the mercaptide in water and remove the copper by passing in a stream of H_2S. Filter off the copper sulfide, washing the residue with dilute HCl. Concentrate the filtrate and washings to 500 ml., then decolorize by boiling with 3 g. of charcoal and filtering. Concentrate the filtrate in vacuo under a stream of nitrogen to incipient crystallization. Cool in the refrigerator. An almost solid mass of cysteine hydrochloride should form. Filter off the crystals and wash with cold concentrated HCl.

Preparation of Cystine by Schmidt's Method. Human hair or wool which has been freed from oil by extraction with gasoline is hydrolyzed by heating at 100° C. with twice its weight of concentrated HCl. It requires about 12 hours to effect complete hydrolysis. The mixture must not be heated for any length of time beyond the point at which the biuret test is either negative or feebly positive since cystine is destroyed during the process of hydrolyzing the protein. The greater part of the hydrochloric acid is removed by distilling in vacuo at a temperature between 60° to 70° C. and the original volume of the solution is restored by the addition of water. A thick aqueous suspension of commercial finishing lime is now slowly added, care being taken to avoid any considerable rise in temperature, until the mixture has acquired a chocolate color. It is then filtered by suction through a Buchner funnel and the residue washed a number of times with distilled water. The filtrate should be clear and possess a light brown color. Hydrochloric acid is now added to partially neutralize the alkaline solution and it is finally acidified by addition of acetic acid. On standing over night in the icebox, sedimentation of the crude cystine takes place. This is filtered off and is dissolved in a minimum quantity of 5 per cent HCl. The solution is decolorized by boiling for several minutes with a small quantity of charcoal which has been previously boiled with HCl to remove the calcium phosphate, and the cystine is precipitated by the addition of sodium acetate to the hot solution until a drop of the solution ceases to turn Congo-red paper blue. The mixture is filtered at once and the cystine is washed a number of times with hot water to completely remove the last traces of tyrosine. Typical hexagonal plates of cystine are obtained. (See Fig. 45.)

Tests for Cystine and Cysteine.

(a) SULLIVAN'S TESTS.

Cystine. To 5 ml. of the solution under test (containing not more than 0.04 per cent of cystine in approximately 0.1 N hydrochloric acid, at a temperature of about 25° C.) add 1 or 2 ml. of freshly made 5 per cent aqueous solution of sodium cyanide. Mix and let stand 10 minutes. Then add 1 ml. of a freshly prepared 0.5 per cent solution of 1,2-naphthoquinone-4-sodium sulfonate, sodium sulfite, etc., as given below for cysteine.

Cysteine. To 5 ml. of solution containing not more than 0.04 per cent of cysteine in 0.1 N hydrochloric acid, add 1 ml. of 1 per cent sodium cyanide in 0.8 N sodium hydroxide. Mix and add 1 ml. of a freshly prepared 0.5 per cent aqueous solution of 1,2-naphthoquinone-4-sodium sulfonate. Mix and add 5 ml. of 10 to 20 per cent solution of anhydrous sodium sulfite in 0.5 N sodium hydroxide. Mix and let stand 30 minutes. A reddish-brown color appears. Then add 1 ml. of a 2 per cent solution of sodium hyposulfite ($Na_2S_2O_4$) in 0.5 N sodium hydroxide. The brown-red color in the presence of cysteine (or cystine treated with sodium cyanide) is converted to a purer red.[9]

(b) TESTS FOR SULFHYDRYL (SH) GROUP. On the addition of a dilute solution of $FeCl_3$, an indigo-blue color appears and disappears almost immediately. Add a dilute solution of $CuSO_4$, whereupon a transitory violet color appears.

Test 1 or 2 ml. of cysteine solution with a dilute solution of sodium nitroprusside and a drop of NaOH. A deep purple-violet color appears but gradually fades after a few minutes.

Add a few drops of a 10 per cent aqueous solution of lead acetate to the cystine (or cysteine) solution, then render the solution strongly alkaline with 40 per cent NaOH or KOH. Boil for a few minutes. If cystine or cysteine are present, the solution becomes brown and a black precipitate of PbS appears (lead-blackening test).

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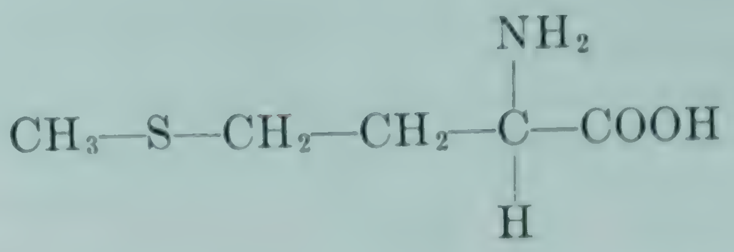
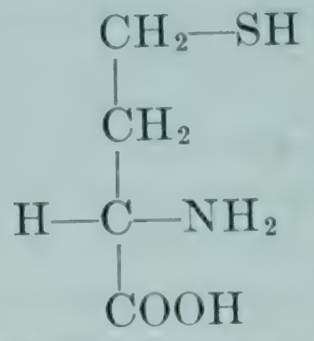
[9] The reaction requires a high final pH. In case of hydrolyzates of foodstuffs it is necessary to add 1 or 2 ml. of 5 N sodium hydroxide just before adding the final reducing agent, sodium hyposulfite ($Na_2S_2O_4$). As a rule it is cystine that is found in hydrolyzates.

L(−)-Methionine, $C_5H_{11}O_2NS$ (*γ-methylthiol-α-amino-n-butyric acid*).



$$CH_3—S—CH_2—CH_2—\overset{NH_2}{\underset{H}{C}}—COOH$$

When methionine is treated with concentrated sulfuric acid, the methyl group is split off and the amino acid homocysteine is ultimately obtained.



$$\begin{array}{c} CH_2—SH \\ | \\ CH_2 \\ | \\ H—C—NH_2 \\ | \\ COOH \end{array}$$

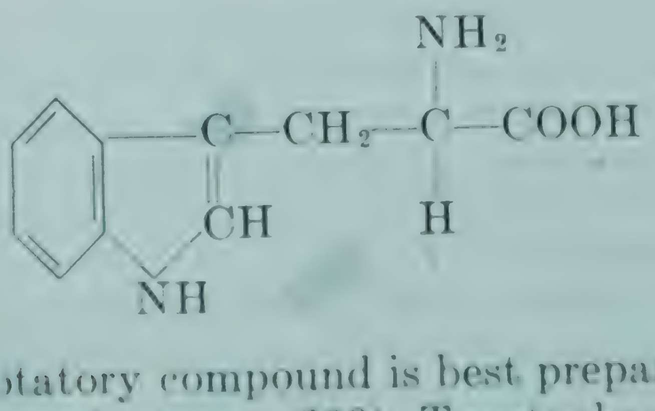
Homocysteine

Homocysteine is similar to cysteine in many ways, and its formation and quantitative determination may be used for the quantitative determination of methionine. While homocysteine has not as yet been isolated from proteins, there is good evidence that it is an intermediate in the biological transformation of methionine into cystine (see p. 1030).

EXPERIMENT ON METHIONINE

Methionine can be detected in a mixture of amino acids by the following method (Bolling's modification of McCarthy-Sullivan). **To 7.5 ml. of unknown, add the following reagents in order, mixing after each addition: 1.5 ml. of 5 N NaOH, 1.5 ml. of 1 per cent glycine, and 0.3 ml. of 10 per cent sodium nitroprusside (freshly prepared). Place the tube in a water bath at 37° to 40° C. for 15 minutes, cool in ice water for 5 to 7 minutes, and add 3 ml. of 6 N HCl. Shake for 1 minute and let stand at room temperature for 15 minutes. A reddish-purple color indicates methionine. Tryptophan interferes.**

L(−)-Tryptophan, $C_{11}H_{12}O_2N_2$ (*β-3-indole-α-aminopropionic acid*).



The natural levorotatory compound is best prepared by the prolonged action of trypsin on casein (see p. 398). Tryptophan is present in nearly all proteins, but is absent from gelatin and zein.

EXPERIMENTS ON TRYPTOPHAN

Color Reactions for Tryptophan. (a) HOPKINS-COLE TEST. **If a substance containing this amino acid is placed in a test tube, a solution containing a**

small amount of glyoxylic acid added, and sulfuric acid then stratified on the bottom of the tube, a reddish-violet ring will appear at the juncture of the two liquids. This is also known as the glyoxylic acid test (see p. 170). Pure tryptophan will not give this test except in the presence of a trace of ferric or cupric ions.

(b) ALDEHYDE REACTION (VOISENET-RHODE). **Tryptophan will also give color reactions in the presence of aromatic aldehydes. With *p*-dimethylaminobenzaldehyde in sulfuric acid it gives a red-violet color. These color reactions are apparently due to the presence of the indole ring in tryptophan. The indole ring, being a combination of the benzene and pyrrole rings, probably owes its chromogenic properties to the latter ring. For other tryptophan reactions see Chapter 16.**

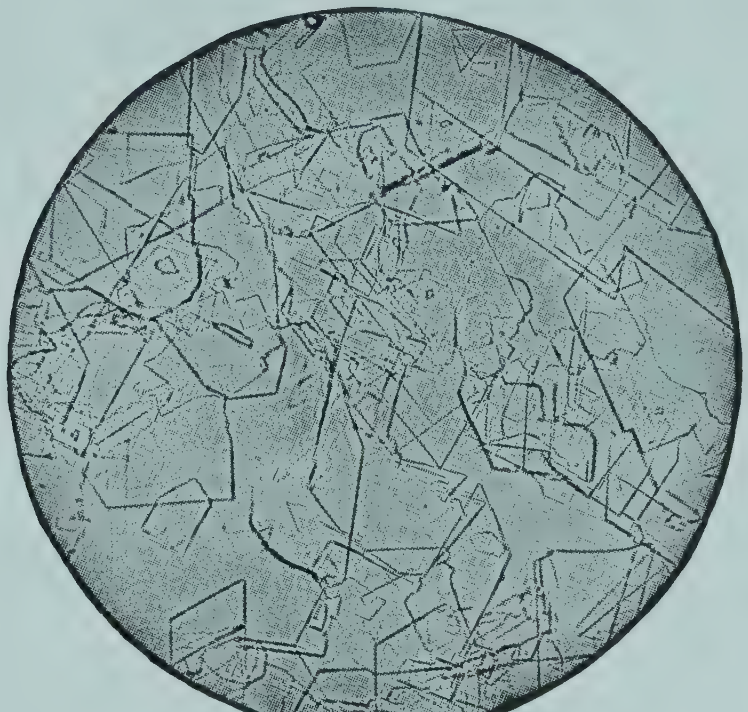
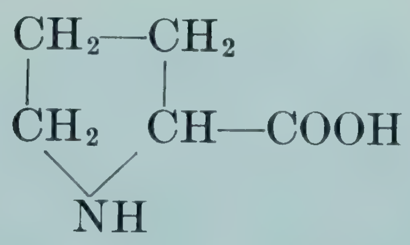


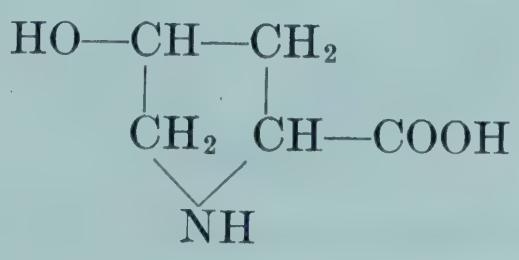
FIG. 46. TRYPTOPHAN.
From Keenan: *J. Biol. Chem.*, **62**, 163 (1924).

L(—)-Proline, $C_5H_9O_2N$ (*pyrrolidine-2-carboxylic acid*).



Proline is easily soluble in alcohol and in cold water. It has a sweet taste and melts at 153° to 154°.

L(—)-Hydroxyproline $C_5H_9O_3N$ (*4-hydroxy-pyrrolidine-2-carboxylic acid*).



It is very difficult to separate hydroxyproline from the other amino acids. It is easily soluble in water and soluble with difficulty in absolute alcohol.

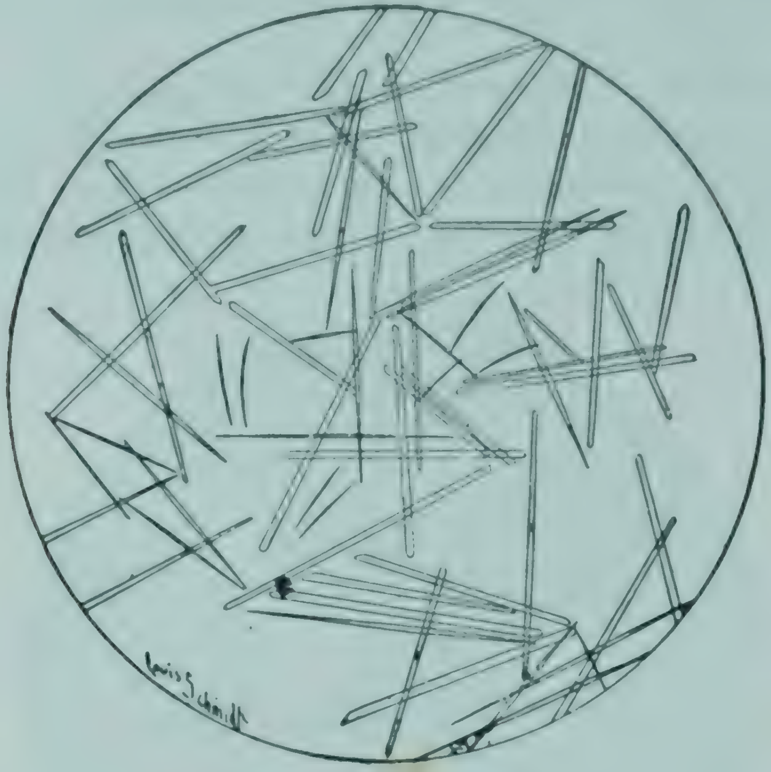
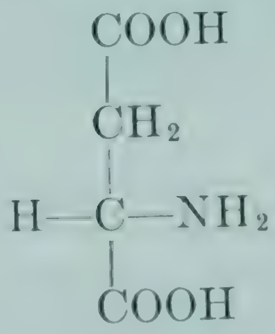


FIG. 47. PROLINE.

L(−)-Aspartic acid, $C_4H_7O_4N$ (*α-aminosuccinic acid*).



$$\begin{array}{c} \mathrm{COOH} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{H}—\mathrm{C}—\mathrm{NH_2} \\ | \\ \mathrm{COOH} \end{array}$$

This amino acid, unlike the others thus far considered, is strongly acidic on account of the predominance of the carboxyl group. The chief source

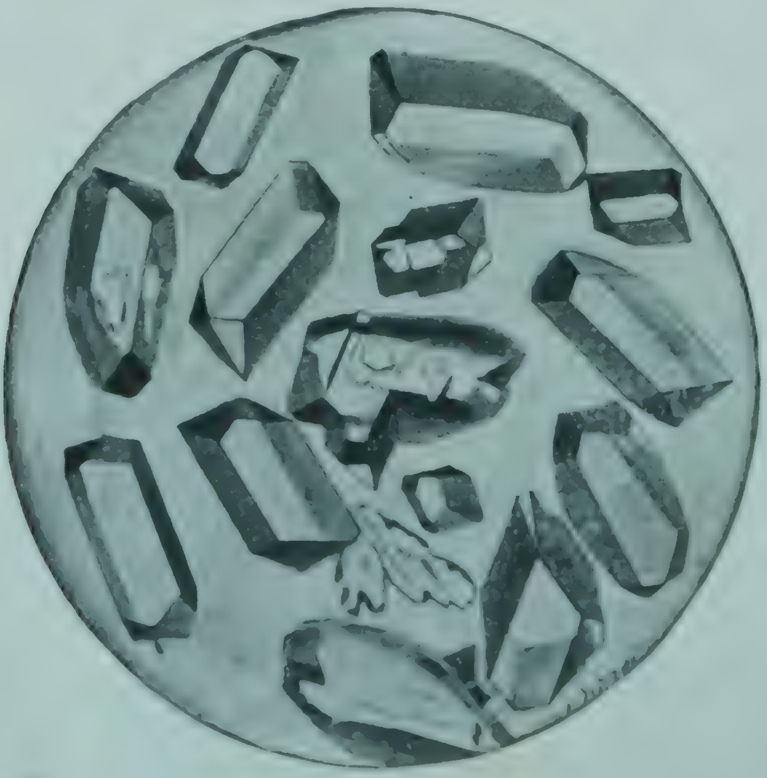
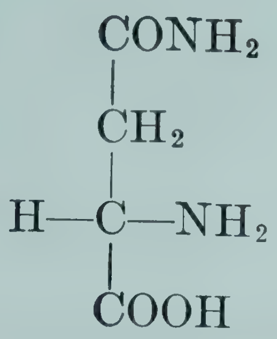


FIG. 48. ASPARTIC ACID.

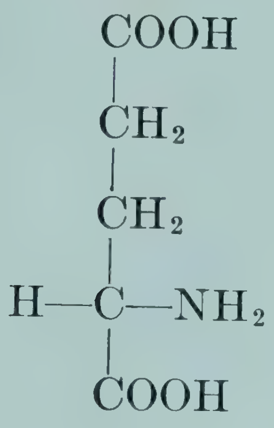
of this acid is the monoamide, asparagine, which is very widely distributed in the vegetable world, being particularly abundant in the asparagus plant and in lentil sprouts. It is more than likely that the amide (asparagine) rather than aspartic acid occurs in the protein molecule, but is quickly hydrolyzed into aspartic acid and ammonia during the process of protein cleavage.



$$\begin{array}{ccc} & \mathrm{CONH_2} & \\ & | & \\ & \mathrm{CH_2} & \\ & | & \\ \mathrm{H}- & \mathrm{C} & -\mathrm{NH_2} \\ & | & \\ & \mathrm{COOH} & \end{array}$$

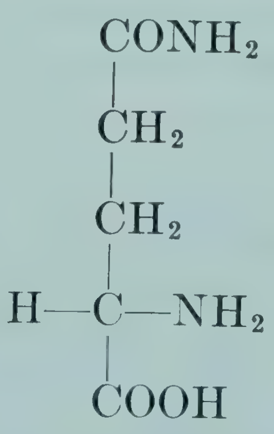
Asparagine

L(+)-Glutamic acid, $C_5H_9O_4N$ (*α-aminoglutaric acid*).



$$\begin{array}{ccc} & \mathrm{COOH} & \\ & | & \\ & \mathrm{CH_2} & \\ & | & \\ & \mathrm{CH_2} & \\ & | & \\ \mathrm{H}- & \mathrm{C} & -\mathrm{NH_2} \\ & | & \\ & \mathrm{COOH} & \end{array}$$

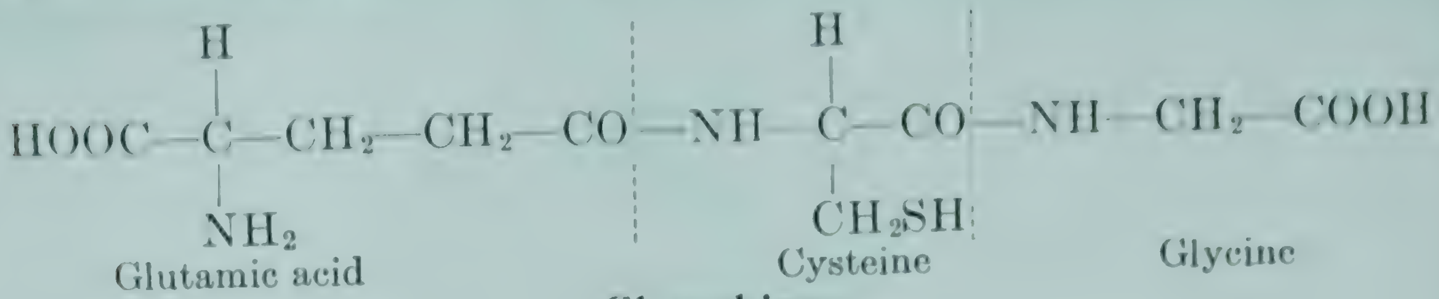
This acid, when obtained after the hydrolysis of the protein molecule, is largely a secondary product. The primary constituent of the protein molecule is undoubtedly glutamine, the amide of glutamic acid, which accounts for the greatest part of the total glutamic acid found after hydrolysis of the protein. Glutamic acid is present in practically all proteins, usually in fairly large amounts, being present to the extent of 47 per cent in gliadin, a protein found in wheat, and to the extent of 23.3 per cent in casein. The sodium salt of glutamic acid is widely used commercially for flavoring soups, sauces, and food concentrates.



$$\begin{array}{ccc} & \mathrm{CONH_2} & \\ & | & \\ & \mathrm{CH_2} & \\ & | & \\ & \mathrm{CH_2} & \\ & | & \\ \mathrm{H}- & \mathrm{C} & -\mathrm{NH_2} \\ & | & \\ & \mathrm{COOH} & \end{array}$$

L(+)-Glutamine

Glutamic acid is found combined with glycine and cysteine in the glutathione molecule (see p. 321). Glutathione has been shown to be a tripeptide, having the following structure:



$$HOOC-\underset{NH_2}{\overset{H}{C}}-CH_2-CH_2-CO-NH-\underset{CH_2SH}{\overset{H}{C}}-CO-NH-CH_2-COOH$$

Glutathione

Attention is called to the fact that the glutamic acid is linked to the amino group of cysteine through its γ carboxyl group.

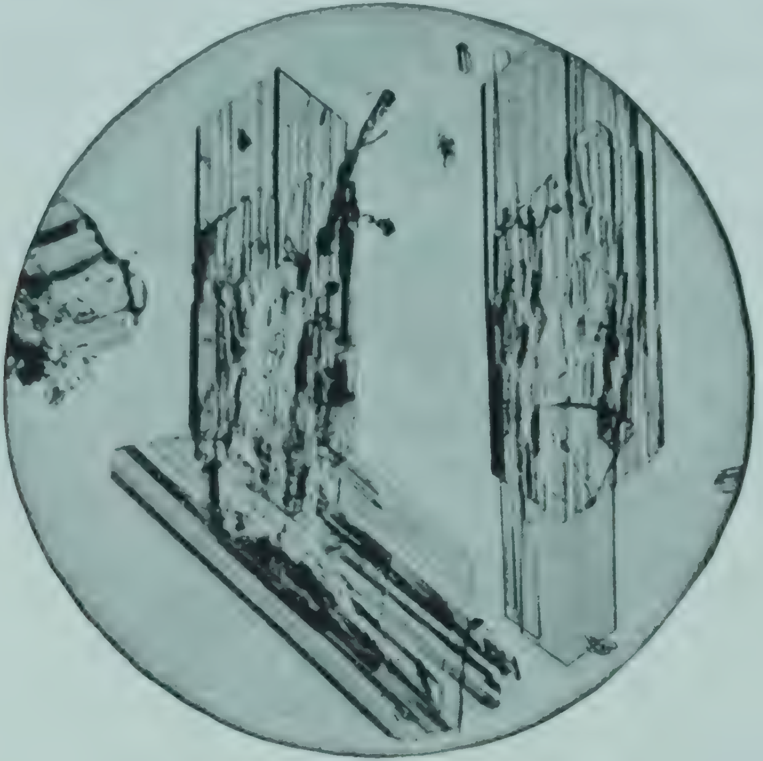


FIG. 49. GLUTAMIC ACID.

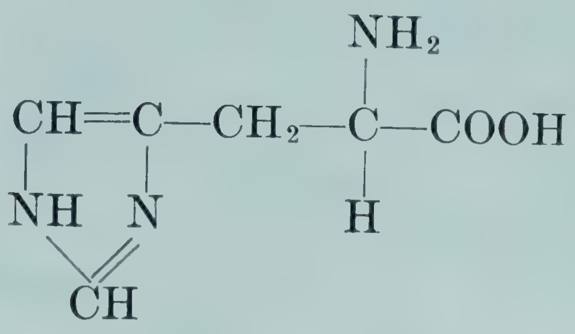
Reproduced from a photomicrograph made by Prof. E. T. Reichert, of the University of Pennsylvania.

EXPERIMENTS ON GLUTAMIC ACID

Preparation of Glutamic Acid. In a 500-ml. flask place 100 g. of gluten flour and 300 ml. of concentrated HCl. Heat on a water bath until the gluten has dissolved. Boil under a reflux condenser for 8 hours on a wire gauze. Cool, dilute with an equal volume of water, and filter. Evaporate to about one-fourth the volume in vacuo. Transfer the residue to a 250-ml. Erlenmeyer flask and saturate with HCl gas, then cool, seed, and place in the icebox for 2 to 3 days. By this time crystals of glutamic acid hydrochloride should have formed. Add an equal volume of ice-cold acetone and filter through a sintered glass funnel. Instead of recrystallizing the crystals, wash in the funnel with ice-cold acetone and ether.

In order to prepare the free glutamic acid from the glutamic acid hydrochloride, dissolve the crystals in the least possible amount of hot water, and then add normal alkali until the solution no longer gives a blue coloration with Congo red, pH 3.2-3.3. Evaporate the solution to 60 ml. in vacuo at 40°. Allow this solution to remain in the icebox until crystallization is complete. Filter off the crystals, wash with a little cold water, and dry. Determine the melting point of the crystals. Examine microscopically (see Fig. 49).

L(—)-Histidine, $C_6H_9O_2N_3$ (*β-imidazole-α-aminopropionic acid*).



Histidine is found in small quantities in practically all proteins, but is present to the extent of 8 per cent in hemoglobin. It combines with a variety of substances, forming, for example, mono- and dihydrochlorides, also compounds or double salts with platinum chloride or silver nitrate which are particularly valuable for obtaining a pure crystalline compound. It also forms characteristic compounds with picric acid, phosphotungstic acid, nitrobenzoyl chloride, 2,5-dichlorobenzene sulfonic acid, nitranilic acid, etc.

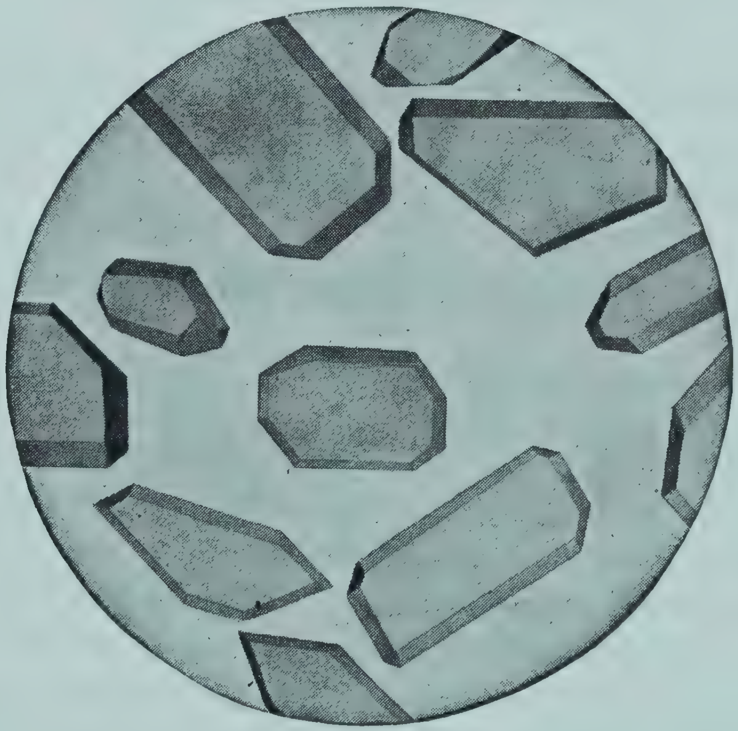
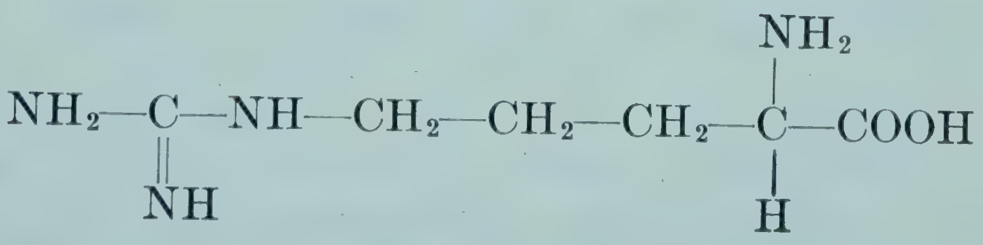


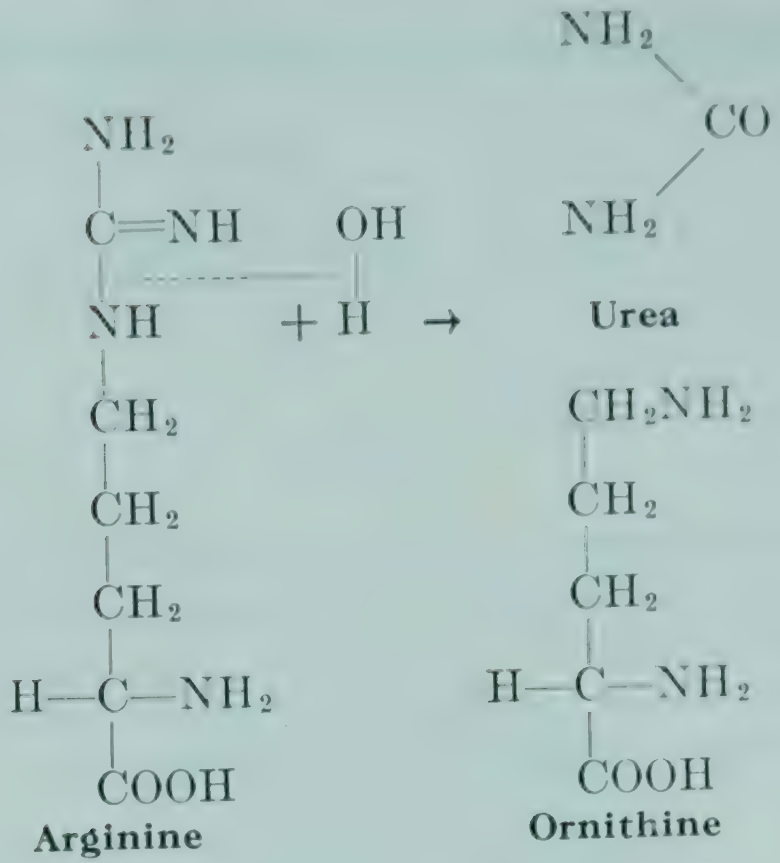
FIG. 50. HISTIDINE DIHYDROCHLORIDE.

L(+)-Arginine, $C_6H_{14}O_2N_4$ (*δ-guanidino-α-aminovaleric acid*).



$$NH_2-\underset{\underset{NH}{\|}}{C}-NH-CH_2-CH_2-CH_2-\underset{\underset{H}{|}}{\overset{\overset{NH_2}{|}}{C}}-COOH$$

Arginine is present in all tissue proteins. It is hydrolyzed into urea and the amino acid ornithine (α,δ-diaminovaleric acid) by the enzyme arginase which is found in the liver.

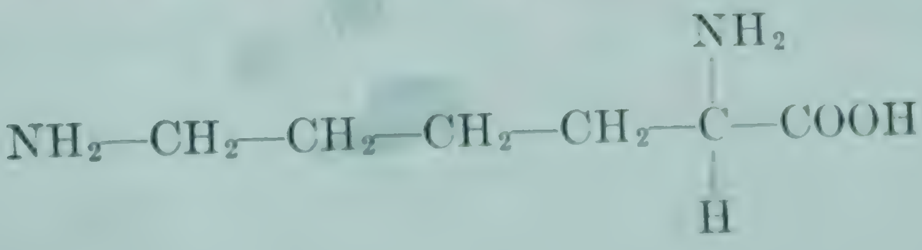


This reaction is thought to be of major importance in connection with the formation of urea by the body (see Chapter 33 for a discussion of this and other metabolic relationships of arginine).

EXPERIMENTS ON ARGININE

1. Isolation of Arginine Flavianate **(Kossel).** Hydrolyze a 25-g. portion of gelatin by boiling with 250 ml. of 18 per cent hydrochloric acid under reflux for 18 hours. Remove the excess acid by repeated concentration in vacuo. Take up the residue in 250 ml. of hot water and decolorize with 5 g. of charcoal. Bring the filtrate to a volume of 250 ml. and add a saturated aqueous solution containing 200 g. of flavianic acid (2,4-dinitro-1-naphthol-7-sulfonic acid) at room temperature. Allow the precipitate to form in the cold for 5 days, stirring from time to time. Filter off the yellow precipitate and wash with a little cold water. Dissolve the washed precipitate in hot water with the aid of a minimal quantity of 4 per cent ammonia. While the solution is still hot add sufficient 20 per cent hydrochloric acid to neutralize all the ammonia. Arginine flavianate crystallizes in shining yellow plates from the hot solution.

L(+)-Lysine, $C_6H_{14}O_2N_2$ (*α-ε-diaminocaproic acid*).



$$NH_2-CH_2-CH_2-CH_2-CH_2-\overset{NH_2}{\underset{H}{C}}-COOH$$

Lysine is one of the basic amino acids, possessing a predominance of amino groups over acidic or carboxyl groups. Histidine, arginine, and lysine have been called "the hexone bases." Lysine is present in most proteins of animal origin. It is notably absent from zein and present in rather small amounts in gliadin.

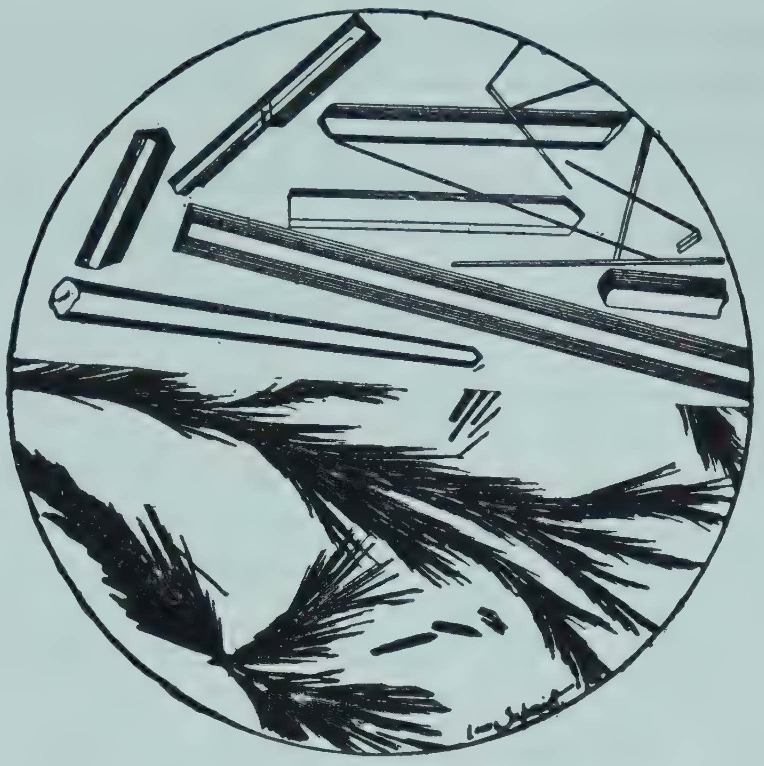
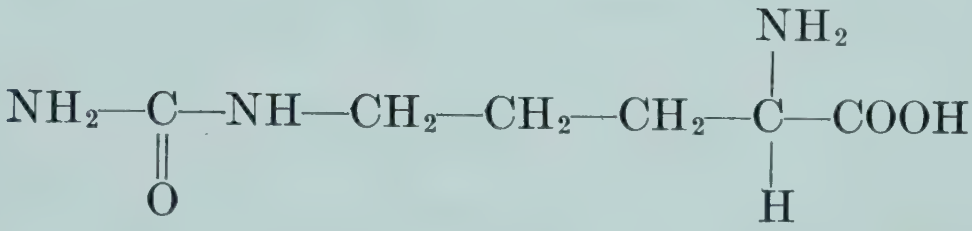


FIG. 51. LYSINE PICRATE.

Citrulline, $C_6H_{13}O_3N_3$ (*δ-carbamino-α-aminovaleric acid*).



$$\mathrm{NH_2{-}\underset{\overset{\|}{O}}{C}{-}NH{-}CH_2{-}CH_2{-}CH_2{-}\underset{\underset{H}{|}}{\overset{\overset{NH_2}{|}}{C}}{-}COOH}$$

Citrulline was obtained by Wada in 1930 from watermelon juice. Little is known concerning its distribution in proteins. Free citrulline is present in small amounts in liver and in blood. Interest in citrulline at the present time is based largely upon its relation to arginine and the processes of urea formation in the animal body (see Chapter 33).

Newer Amino Acids. The application of newer research techniques such as paper chromatography for the isolation and identification of amino acids in hydrolyzates, and the use of microorganisms as nutritional test subjects, have led to the discovery of a number of amino acids and related compounds not hitherto identified among the products of protein hydrolysis. Among those of biological interest, mention may be made of:

α-Aminobutyric acid found in the brain and central nervous system, where it is formed by decarboxylation of glutamic acid.[10] It has also been shown to be produced enzymatically from DL-threonine.[11]

Lanthionine (THIODIALANINE) from wool;[12]

Cystathionine, a condensation product of homocysteine and serine intermediate in the synthesis of cysteine;[13]

Ergothioneine, found in ergot, blood (1.8–1.95 mg. per 100 ml. in human blood),[14] and in the seminal plasma of the boar;[15]

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[10] Roberts and Frankel: *J. Biol. Chem.*, **187**, 55 (1950); **188**, 789 (1951); **190**, 505 (1951).

[11] Lien and Greenberg: *J. Biol. Chem.*, **195**, 637 (1952); **200**, 367 (1953).

[12] Horn, Jones, and Ringel: *J. Biol. Chem.*, **138**, 141 (1941).

[13] Binkley, Anslow, and du Vigneaud: *J. Biol. Chem.*, **143**, 559 (1942).

[14] Hunter: *Biochem. J.*, **48**, 265 (1951).

[15] Mann and Leone: *Biochem. J.*, **53**, 140 (1953).

3,5,3′-Triiodothyronine from thyroid glands;[16]

Methionine sulfoximine, the toxic factor isolated from flour matured by treatment with nitrogen trichloride;[17]

Tabtoximine (α,ϵ-DIAMINO-β-HYDROXYPIMELIC ACID), a phytotoxic compound found in certain diseased tobacco plants.[18]

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[16] Gross and Pitt-Rivers: *Biochem. J.*, **53,** 645 (1953).

[17] Bentley, et al.: *Nature*, **164,** 438 (1949).
Reiner, et al.: *J. Am. Chem. Soc.*, **72,** 229 (1950).

[18] Woolley, Schaffner, and Braun: *J. Biol. Chem.*, **198,** 807 (1952).

5

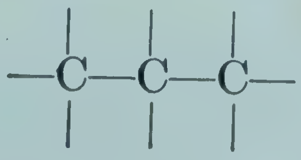
Proteins: Their Structure and General Reactions

The results of investigations on the hydrolysis of proteins indicate that the protein molecule is composed almost exclusively, if not entirely, of α-amino acids. Investigations of this type, which involve the complete tearing down of the protein molecule, yield the constituent amino acids but throw practically no light on the question as to the order in which these acids are linked together to form the intact protein molecule with its characteristic chemical, physicochemical, colloidal, and biological properties. In spite of this defect these researches are of the highest importance since they yield the actual "building stones" of the protein molecule.

In recent years emphasis has been placed on the study of the physicochemical behavior of the intact protein. The difficulty involved in the formulation of an acceptable theory for the structure of the protein molecule becomes apparent when we list the great number and variety of properties and reactions of proteins that such a theory is called upon to explain. Of these, we may mention especially the following: (1) The hydrolysis of proteins by acids, alkalies, and proteolytic enzymes into their constituent amino acids; (2) the small proportions of free amino and carboxyl groups in the intact molecule; (3) the large increase in both amino and carboxyl groups that accompanies the hydrolysis of the protein; (4) the combinations of proteins with acids, bases, and many other classes of compounds; (5) the large size and colloidal nature of the protein molecule, together with the complex colloidal behavior of protein solutions; (6) the complex solubility relationships of the various classes of proteins; (7) the sensitiveness of proteins to chemical and physical agents such as acids, alkalies, alcohol, heat, mechanical shaking, ultraviolet light, etc.; (8) the immunological reactions of the proteins; and (9) their highly specific enzymatic, hormonal, and other biological properties. In the following sections the modern conceptions of protein structure will be discussed briefly together with the more important reactions and properties of proteins which make them such vital constituents of living protoplasm.

Structure of Protein Molecule: Peptide Linkage. It was pointed out by Hofmeister, in 1902, that there are three conceivable types of linkage by which individual amino acids might be joined together in the protein molecule.

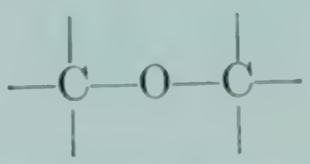
The first type of linkage involves direct union between carbon atoms:



$$-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-$$

This type of union is very unlikely since bonds between carbon atoms are not attacked by proteolytic enzymes which hydrolyze native proteins. It is difficult, also, to understand how a molecule having such a structure could be broken down by hydrolytic agents into such definite structural units as the polypeptides and amino acids.

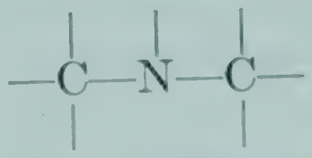
The second type of linkage that suggests itself is a linkage of carbon atoms by means of an oxygen atom, as in the ethers, esters, and anhydrides:



$$-\overset{|}{\underset{|}{C}}-O-\overset{|}{\underset{|}{C}}-$$

This type of union is also improbable since, in view of the number of its carboxyl groups, the protein molecule does not contain sufficient oxygen to account for a major linkage of that kind. Moreover, since such a linkage does not involve the amino groups, the latter should be much more abundant in the intact protein molecule than they actually are.

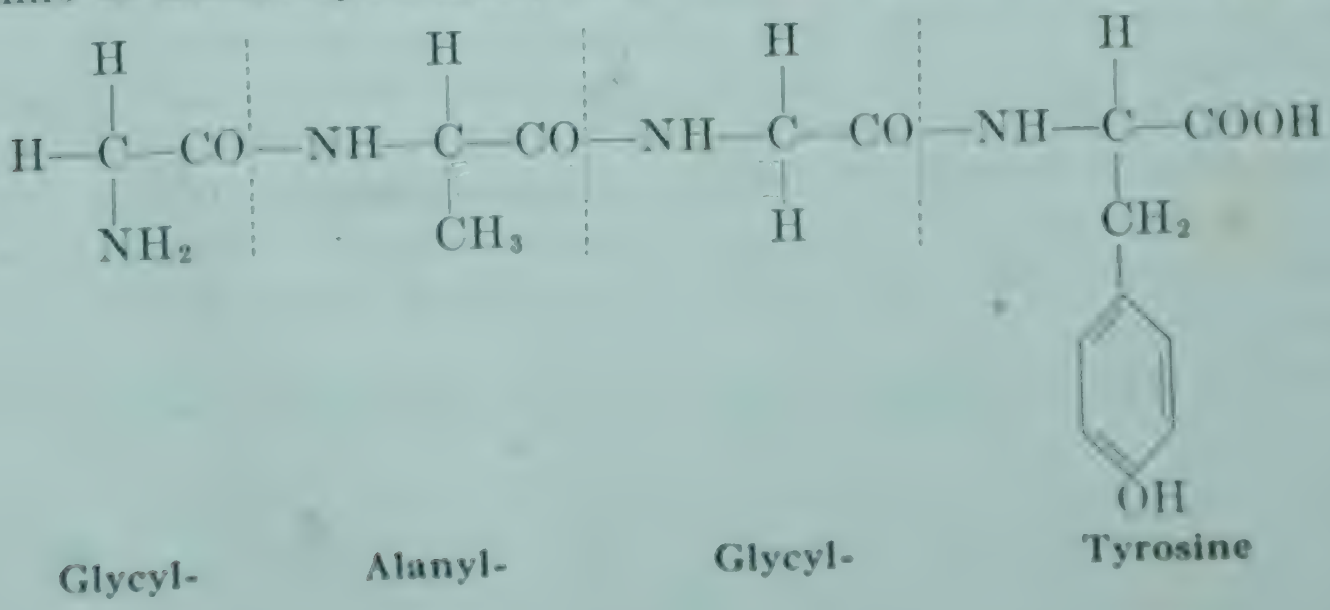
This leaves, as the final possibility, the linkage of carbon atoms by means of a nitrogen atom:



$$-\overset{|}{\underset{|}{C}}-\overset{|}{N}-\overset{|}{\underset{|}{C}}-$$

Of the various possibilities for such a linkage, that resulting from the condensation of the amino group of one acid with the carboxyl group of another, which Fischer named the *peptide linkage*, is the only one which is in accord with the experimental facts. Fischer devised several ingenious methods for condensing amino acids in this manner and prepared a large number of di-, tri-, and polypeptides, some of them containing as many as 18 molecules of amino acids. Many of the synthetic polypeptides prepared by Fischer, Bergmann, and others are identical with polypeptides isolated from partially hydrolyzed proteins.

According to modern conceptions of protein structure the peptide bond is the predominant bond in the protein molecule. This point of view is based on a great mass of experimental evidence, of which only a brief review may be given here. The structural formula of the tetrapeptide given below indicates clearly that only those amino and carboxyl groups which are at the ends of chains are free. When the molecule contains diamino or dicarboxylic acids, the additional amino or carboxyl groups



either may remain free or may be the starting points for side chains of various kinds. Analyses of proteins indicate that the number of free amino and carboxyl groups in the intact molecule, determined by Van Slyke's nitrous acid and Sørensen's formol titration methods, is substantially what would be expected of a molecule built up of amino acids joined together by means of peptide linkages. Furthermore, hydrolysis of proteins by acids or enzymes results in the liberation of equal numbers of amino and carboxyl groups, such as would arise during the hydrolysis of peptide bonds. The biuret reaction, which is characteristic of proteins and some of their decomposition products, is given by many of the synthetic polypeptides and practically disappears when all these substances are completely hydrolyzed.

Relation of Structure to Properties of Proteins. The generally accepted theory of Hofmeister and Fischer that proteins consist of chains of amino acids joined to each other through their amino and carboxyl groups does not by itself seem to explain the unique chemical, physical, and biological differences among proteins in nature. Why are keratins so resistant to dilute acids and proteolytic enzymes? How does the muscle protein, myosin, function in the contraction of muscle? What is responsible for the immunological specificity of proteins, for the enzyme action of catalase and pepsin, for the toxicity of tobacco mosaic virus, and so forth? The hypotheses which are mentioned briefly below are attempts to answer some of these questions.

A. PROTAMINE NUCLEUS HYPOTHESIS OF KOSSEL AND SIEGFRIED. These investigators believed that all proteins were built around a nucleus of the three amino acids arginine, histidine, and lysine, and that arginine was the most important member of this triad. Block has shown that one group of proteins, eukeratins, can be characterized by the relative constancy of the ratios of arginine to lysine to histidine. The location of these three amino acids or their mode of action in the formation of the eukeratins remains unknown. It should also be pointed out that no protein devoid of arginine has as yet been reported, although some large peptides are lacking in this amino acid.

B. DIKETOPIPERAZINE HYPOTHESIS OF ABDERHALDEN. Amino acid anhydrides (diketopiperazines, see p. 130) can be obtained from proteins under certain conditions. These compounds are readily formed by condensation of two amino acids or by cyclizing a dipeptide. Abderhalden suggested that proteins are composed of diketopiperazine-containing units which are held together by secondary valences. This structure was intended to account for certain properties of proteins, especially the hydrolysis to peptones by pepsin. Unfortunately for this theory, amino acid anhydrides are not hydrolyzed by proteolytic enzymes.

C. STEREOCHEMICAL ORGANIZATION OF THE PROTEIN MOLECULE (FOA, MEYER AND MARK, ASTBURY, NEURATH, WRINCH, PAULING). The use of the x-ray to study the fine structure of proteins, especially in the hands of W. T. Astbury, has given us a much clearer insight into the organization of the peptide chains in the protein molecule. A short section of such a chain in the fully extended position is shown in Fig. 52. In general these peptide chains are pictured as either fully extended (silk fibroin, or other denatured, mechanically elongated fibrous proteins) or folded chains of (native) insoluble fibrous and soluble globular proteins. In certain cases, such as hair and wool, the long axis of the peptide chain is parallel to the long axis of the fiber, and the strength, elasticity, and resistance to enzymatic hydrolysis can be definitely correlated to this structure. Thus hair keratin can be pictured as consisting of bundles of partially folded peptide chains which are cross-linked to each other through the —S—S— bonds of cystine. The tensile strength of hair is thus explained by the large number of similar peptide chains in parallel; the elasticity by the folding and unfolding of the chains; and the resistance to enzymes by the close packing of the parallel chains.

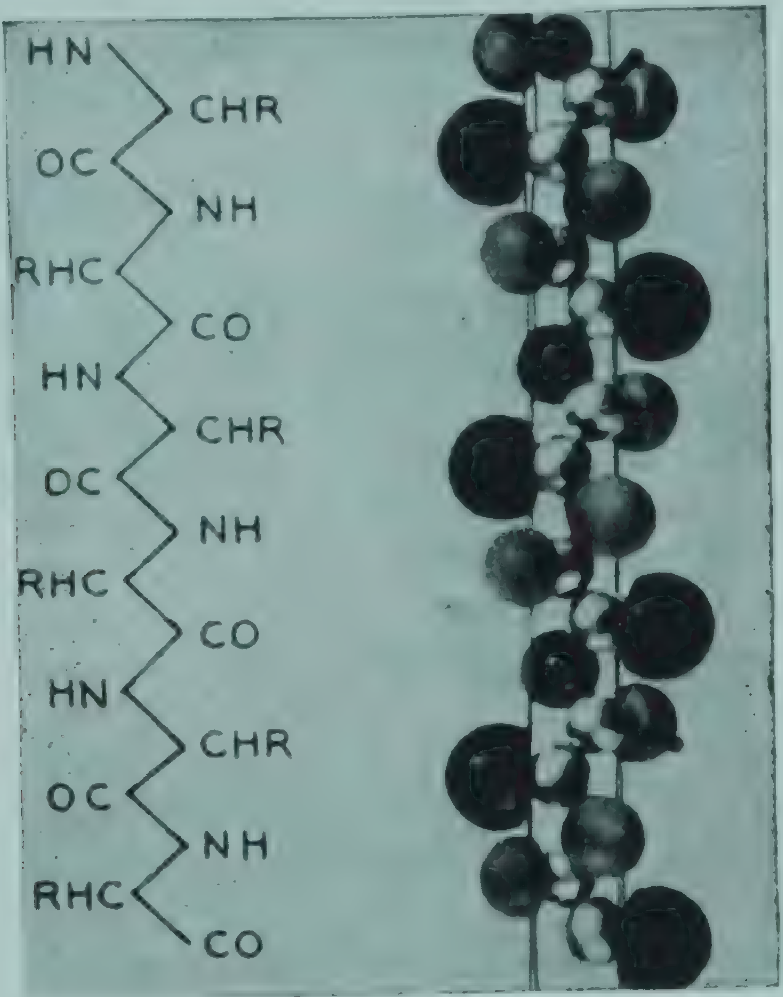


FIG. 52. SCALED MODEL OF A FULLY EXTENDED POLYPEPTIDE CHAIN, VIEWED FROM ABOVE.

Neurath: *J. Phys. Chem.*, **44**, 297 (1940).

The structure of the soluble proteins, especially those whose molecules are essentially oval or globular in shape, is still largely unexplained, although the speculations of Wrinch and others concerning cagelike, spiral, or multiple-discoid structures may be the clues to their ultimate solution.

As mentioned above, it has long been clear that the properties of native proteins cannot be explained solely on the hypothesis that they are formulated as straightforward peptide chains joined by disulfide and other cross linkages (cf. below). Closely similar x-ray diffraction patterns characteristic of α-keratin, β-keratin, and collagen are given by proteins of very different amino acid composition. Thus, it is evident that there must be some regularity of structure of proteins which is independent of the amino-acid side chains and must arise from the coiling or folding of the peptide chains themselves. A number of structures have been proposed to account for these distinctive x-ray patterns, all of which suffer from the disadvantage of not meeting all the experimental evidence. In 1951, Linus Pauling and his co-workers, Corey and Branson, employing as sole restrictions to their formulation the accepted interatomic distances and covalency angles, the planarity of the amide groups, and the linearity of the —N—H------O— hydrogen bond and assuming that the maximum possible number of intramolecular hydrogen bonds would be formed, arrived at certain helical structures.[1] One of these gave 3.7 amino acid residues per complete helical turn. This resulted in 13-membered rings, closed by —CO— and —NH— groups in hydrogen-bond linkage as shown in perspective and in plan (Figs. 53 and 54, respectively). This so-called α-helix has been rather definitely established to occur in hair, horn, and other proteins of the α-keratin class (including muscle) as well as in hemoglobin and many other globular proteins such as serum albumin, insulin, lysozyme, and chymotrypsin. Another proposed helical formation, the γ- or 5.1-residue helix, is now believed to be too unstable to exist in proteins. However, an "antiparallel-chain pleated sheet" structure[1a] appears to be characteristic of silk and stretched hair.

It is generally believed that the gamma globulin of blood is able to assume different shapes under the impact of foreign antigens while retaining its amino acid composition and arrangement unmodified. It seemed that too rigid specification of the peptide (backbone) coiling of gamma globulin would be incompatible with its known lability with respect to the formation of antibodies. The 3.7 amino-acids-residue helix seems capable of overcoming this difficulty since it may be transposed into many different patterns by starting the helix with the same amino acid but at geometri-

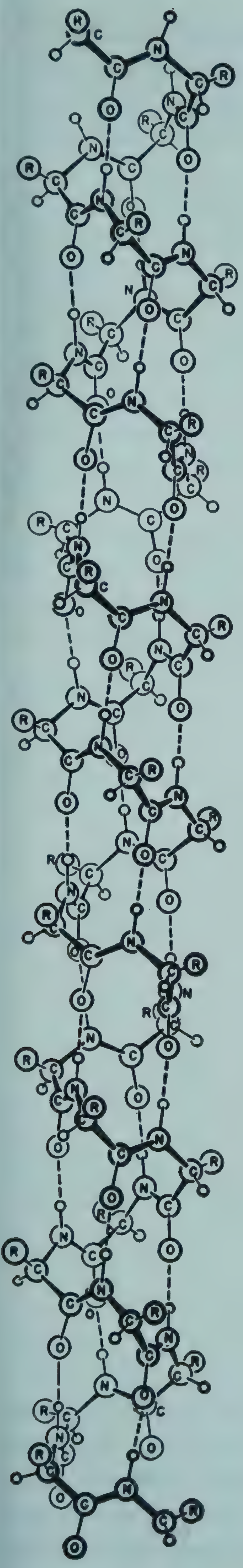


FIG. 53. THE HELIX WITH 3.7 RESIDUES PER TURN.

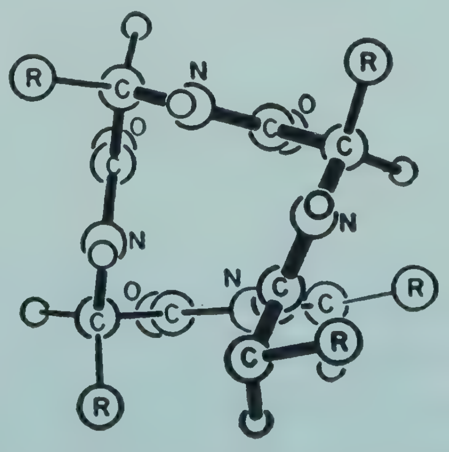


FIG. 54. PLAN OF THE 3.7-RESIDUE HELIX.

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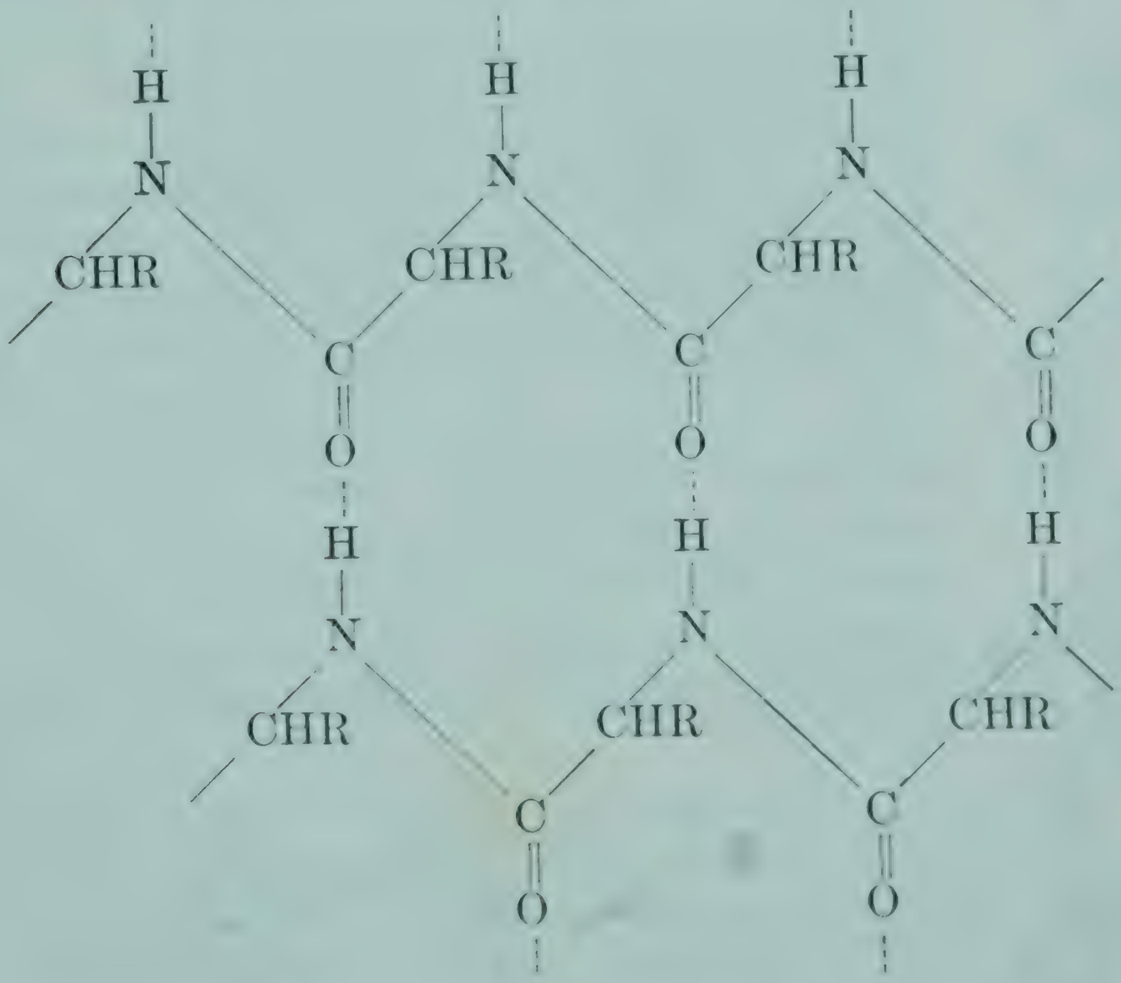
[1] Pauling, Corey, and Branson: *Proc. Natl. Acad. Sci.*, 37, 207 (1951) and personal communication.

[1a] Pauling and Corey: *Proc. Natl. Acad. Sci.*, 37, 729 (1951).

cally different points. To visualize the 3.7 helix better, it may be compared to a spiral staircase with each amino acid residue as a step. The height of each step is 1.5 Å and the height of each turn is 5.4 Å making 3.6 or 3.7 steps per turn. It thus takes 18 steps or 5 turns for a step to be found exactly in a vertical line above the starting point.

The closely knit structure of highly organized soluble globular proteins is broken down by denaturing agents such as strong sodium hydroxide. The peptide chains then become a mass of disorganized fibrils in solution. If this alkaline protein solution is passed through a small capillary, these fibrils orient themselves with their long axes parallel to the direction of flow. If the alkaline solution is then extruded through a small orifice into an acid or other coagulating bath, the protein fibrils unite with each other parallel to their long axes and a typical macroscopic thread is formed. This is the essence of the production of synthetic fiber, "wool," etc., from soluble globular proteins such as casein and soybean.

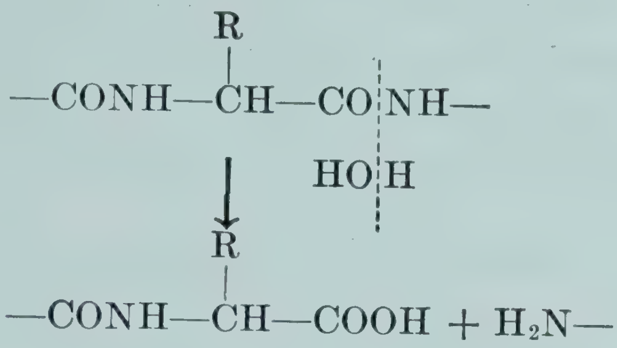
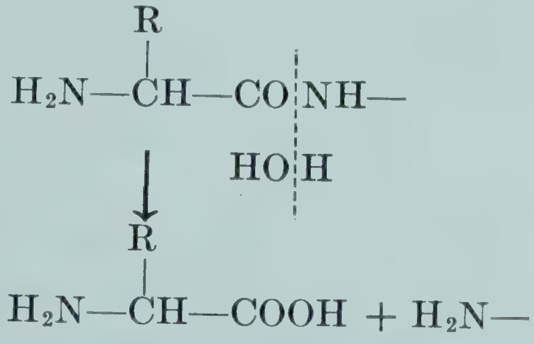
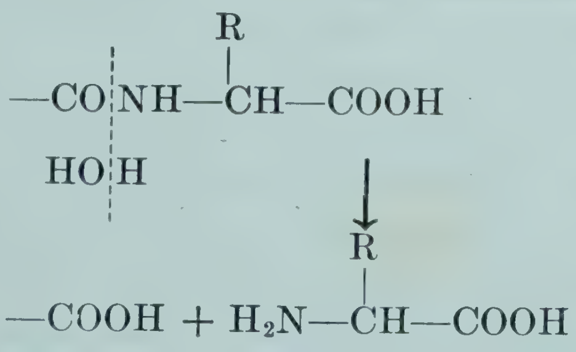
The three main types of cross linkage in proteins appear to be (1) Dithio bridges formed by cystine, (2) Salt bridges formed by the carboxyl groups of aspartic or glutamic acid and by amino groups of lysine or guanido groups of arginine, and (3) Hydrogen bonds between peptide linkages and between polar groups of the amino acids:



From this presentation it is apparent that the solution to the problems of protein structure and function has not yet been found, but that the Hofmeister-Fischer peptide theory, coupled with the known electrical and chemical properties of the peptide linkage (—NH—CO—CHR—) and of the specific side chains of the amino acids, offers a reasonable basis to account for many of the properties of natural and of isolated proteins.

Proteolytic Enzymes. The most convincing evidence that the peptide linkage is the principal type in the protein molecule comes from the fact that enzymes are able to hydrolyze synthetic polypeptides of known structure, liberating equal amounts of amino and carboxyl groups, just as they do when acting on native proteins. Our knowledge on the structural specificity of enzymes is due in large part to the brilliant researches of Bergmann and his co-workers. The table given below illustrates the specificity of enzymes.

Action of Proteolytic Enzymes (Bergmann)

Enzyme	*Requisite Peptide Chain*	*Requisite Amino Acid in Peptide Chain*
Pepsin Cathepsin A(I)	 $-CONH-\overset{R}{CH}-CO\vdots NH- \xrightarrow{HO\vdots H} -CONH-\overset{R}{CH}-COOH + H_2N-$	Tyrosine or Phenylalanine
Trypsin Cathepsin B(II) Papain H_2S	Same as above except for R group	Lysine or Arginine
Leucyl amino-peptidase (Cathepsin III)	 $H_2N-\overset{R}{CH}-CO\vdots NH- \xrightarrow{HO\vdots H} H_2N-\overset{R}{CH}-COOH + H_2N-$	Leucine
Carboxypeptidase (Cathepsin IV)	 $-CO\vdots NH-\overset{R}{CH}-COOH \xrightarrow{HO\vdots H} -COOH + H_2N-\overset{R}{CH}-COOH$	Tyrosine or Phenylalanine

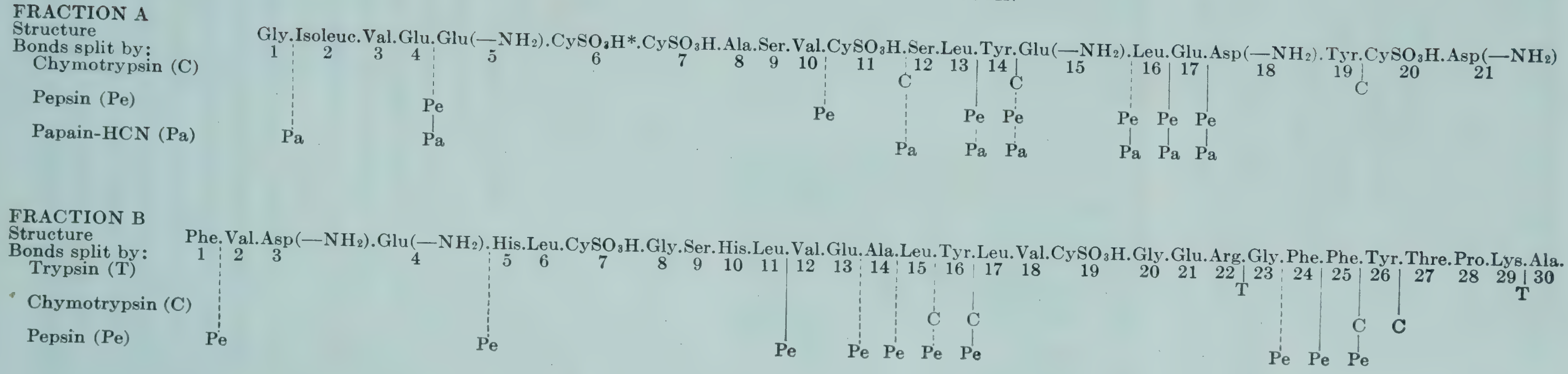
Up to the time of these investigations it was believed that the size and not the amino acid composition of the protein or polypeptide was the controlling factor in enzymatic breakdown. Thus pepsin was thought to act on proteins of high molecular weight such as fibrin, casein, etc., but not on the smaller protamines, while trypsin split the smaller molecules, such as the protamines, and erepsin hydrolyzed peptides of still lower molecular weight. It is now evident that pepsin fails to hydrolyze protamines not because of their molecular size but because these proteins are deficient in tyrosine and phenylalanine, while the ample supply of arginine and lysine in protamines permits the action of trypsin. Likewise a mixture of polypeptides containing a relatively high proportion of free carboxyl groups would be split by carboxypeptidases (erepsin?) such as the "aromatic" carboxypeptidase shown in the table above. In each case, *the ratio of carboxyl to amino groups liberated is unity*. The resistance of certain proteins, such as silk fibroin, hair keratin, etc., to digestion by proteolytic enzymes is not explainable on the basis of amino acid composition but is due to the close packing of the protein molecules, which then offer relatively little surface to the enzyme. If the molecular structure is disorganized by mechanical or chemical means, these proteins are readily hydrolyzed by the proteolytic enzymes.

In the current hypothesis of enzyme action it is assumed that the enzyme-substrate complex is formed by the bonding of complementary surfaces. Thus the mechanism would depend primarily on the steric arrangement of the molecular groups of the substrate, their shape and mutual positions, and only secondarily on the chemical nature of the bond undergoing hydrolysis.

The Composition of Insulin. In spite of all the chemical and physicochemical studies on many proteins, only one protein has been so thoroughly investigated that its amino acid sequence is completely known. In a series of brilliant researches, Sanger and his co-workers at Cambridge, England, have elucidated the chemical structure of pure, crystalline insulin. In brief, they broke the —SS— bonds which hold the peptides of insulin, by oxidation with performic acid. This procedure not only breaks the —SS— bonds but also oxidizes the cysteine residues to cysteic acid residues. The resulting oxidized insulin was then separated into two fractions: fraction A contained only glycine at the amino end of the chain; fraction B contained only phenylalanine as the N-terminal amino acid. These two fractions were then subjected to partial hydrolysis by dilute acid or by enzymes. When the hydrolytic products (amino acids and small peptides) were separated and identified by means of paper chromatography, Sanger, Thompson, and Tuppy were able to fit these together to give the exact sequence of all the amino acids in each of the peptides. Their results are given in the accompanying table.

Molecular Weights of Proteins. Many of the properties of protein solutions, especially those connected with their colloidal behavior, are intimately related to the size and molecular weight of the protein. The determination of the molecular weights of the proteins involves many difficulties owing to the complex solubilities of the proteins and the colloidal nature of their solutions. Thus, for example, the ordinary physicochemical methods, such as the raising of the boiling point or lowering of the freezing point, are either inapplicable or yield misleading results when applied to proteins because of the effects of traces of salts and other impurities on determinations made by those methods. However, the minimal molecular weight can be obtained from chemical, especially amino acid, analyses. By assuming that the protein molecule contains one atom of an element or one molecule of the amino acid present in least amount,

Sequence of Amino Acids in Insulin



FRACTION A

Structure

Gly.Isoleuc.Val.Glu.Glu(—NH₂).CySO₃H*.CySO₃H.Ala.Ser.Val.CySO₃H.Ser.Leu.Tyr.Glu(—NH₂).Leu.Glu.Asp(—NH₂).Tyr.CySO₃H.Asp(—NH₂)

Bonds split by:

Chymotrypsin (C)

Pepsin (Pe)

Papain-HCN (Pa)

FRACTION B

Structure

Phe.Val.Asp(—NH₂).Glu(—NH₂).His.Leu.CySO₃H.Gly.Ser.His.Leu.Val.Glu.Ala.Leu.Tyr.Leu.Val.CySO₃H.Gly.Glu.Arg.Gly.Phe.Phe.Tyr.Thre.Pro.Lys.Ala.

Bonds split by:

Trypsin (T)

Chymotrypsin (C)

Pepsin (Pe)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
C
Pe
Pa
T

| indicates major sites of enzyme action

¦ indicates other bonds split by enzymes

* The cysteic acid exists in the insulin molecule as cystine.

the minimal molecular weight may be calculated from the following relationship:

$$\text{Minimal molecular weight of protein} = 100 \times \frac{\text{Atomic weight of element}}{\text{Percentage of element}} = 100 \times \frac{\text{Molecular weight of amino acid}}{\text{Percentage of amino acid in protein}}$$

On the other hand the relatively large particle size of most proteins has permitted the development of methods for estimating their size and molecular weight which could not be used for the average organic compound. These are:

1. Molecular weight from osmotic pressure (Sørensen, Roche, Adair, Greenberg, et al.).
2. Molecular weight from sedimentation equilibrium and from sedimentation rate and diffusion (Svedberg, Peterson).
3. Molecular weight from diffusion and viscosity (Northrop and Anson, Neurath, et al.).
4. Molecular size and shape from dielectric-constant dispersion curves (Williams, Oncley).
5. Molecular size from x-ray diffraction data (Crowfoot, Fankuchen).

The osmotic-pressure method yields the mean molecular weight when carried out under suitable experimental conditions. This procedure, which can be carried out with ordinary laboratory equipment, suffers from the disadvantages that it gives no indication of homogeneity of the substance investigated and that it becomes increasingly insensitive with increasing molecular weights.

Every molecule in solution is subject to thermal forces which result in diffusion of the substance away from a center of high concentration toward one of lower concentration. In addition to these thermal forces the molecule is subject to gravitational forces which tend to cause the molecules to sediment. It is thus apparent that whether a substance will remain in solution or not is dependent upon the relative strengths of the gravitational (settling) and thermal diffusion forces. By increasing the speeds attainable in the ordinary laboratory centrifuge, Svedberg, Beams, and others have constructed very high-speed centrifuges (ultracentrifuges) which are able either just to balance the diffusion forces of a protein molecule in solution (sedimentation equilibrium) or actually overcome these thermal forces and cause the protein to sediment at a definite rate (sedimentation velocity).

If the concentration, diffusion constant, and approximate shape of the protein are known, the molecular weight or particle size of the protein or other colloid can be calculated either from sedimentation-equilibrium or sedimentation-velocity data (see Fig. 55). One significant contribution of this method is that it is capable of separating particles of different sizes and weights and thus indicating whether the protein under investigation is mono- or polydisperse. Many proteins which were previously considered to be monodisperse are now known to be mixtures of products varying widely in particle size. It should be recalled, however, that protein particles of the same size (monodisperse) may differ in amino acid composition, thus making the protein a mixture; on the other hand protein particles of different sizes (polydisperse) may have the same amino acid composition and thus be chemically pure. Analogies exist in organic chemistry; thus leucine admixed with a little isoleucine would be monodisperse, although impure; whereas acetic acid dissolved in benzene would be both monomeric and dimeric acetic acid (polydisperse) and yet be all the same compound.

Molecular weights of proteins have been calculated from diffusion and viscosity data. Both diffusion and viscosity are functions of the size, shape, and degree of hydration of the molecule.

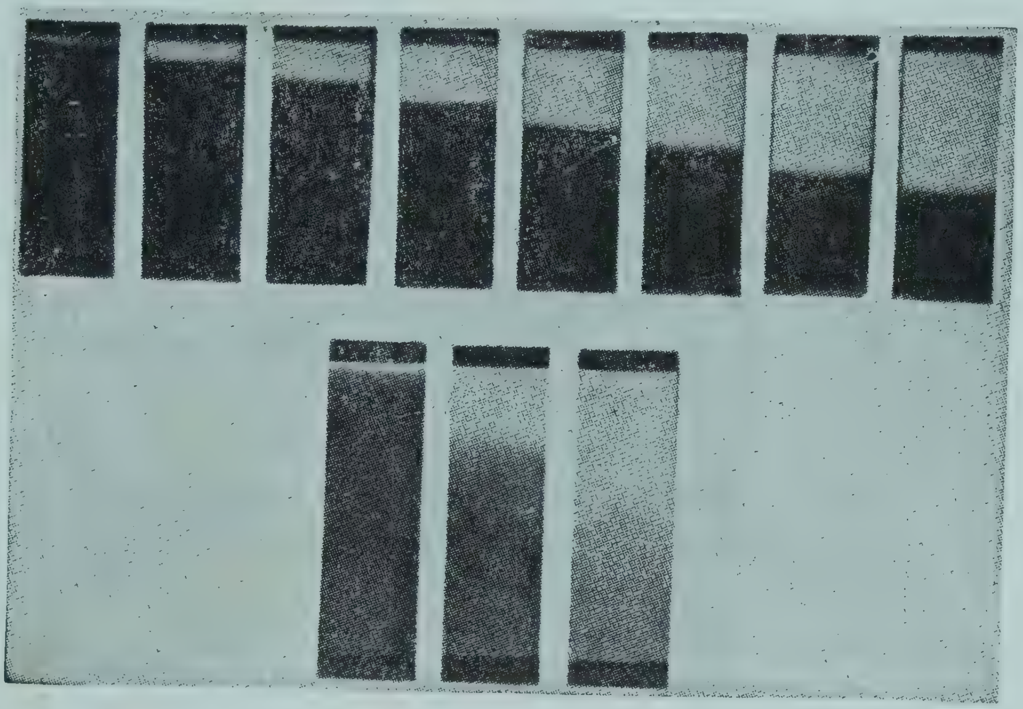


FIG. 55. SEDIMENTATION IN THE ULTRACENTRIFUGE OF A MONODISPERSE SOL (HEMOCYANIN) AND A POLYDISPERSE SOL (GOLD).

From Svedberg: *Colloid Chemistry*, 2nd ed. Reinhold Publishing Corp., New York, 1928.

Proteins, among other substances, can be oriented in an electric field. If the direction of the field is reversed the molecule will reorient in the opposite direction. As the rate of change of direction of the current is increased (i.e., increasing frequency), more and more of the molecules fail to orient properly and the electrical properties of the solution change. The changes in the dielectric properties of a protein solution are also dependent upon the size, shape, and viscosity of the molecule as well as the alternating frequency. Such data have been used to calculate the molecular weight of some proteins.

The final method mentioned above for the estimation of molecular size is based upon the fact that a single protein crystal, when placed in the beam of an x-ray, will cause the beam to be somewhat deflected. From this, the dimensions for a *unit cell* are obtained and from the density of the protein its mass is computed. The molecular weight is calculated by dividing this mass by that of the hydrogen atom (1.66×10^{-24}). The results indicate the molecular weight of the protein or some multiple thereof.

Additional methods for determining molecular weight and shape of proteins are: molecular shape from flow-birefringence data; molecular weight from light scattering of proteins; and dimensions of proteins from electron micrography.

It should be pointed out that values given in the table of molecular weights below may be multiples or fractions of the true molecular weights of the proteins. These methods probably measure the size of the protein particles with considerable accuracy under the particular experimental conditions, but the results do not necessarily indicate the *true* molecular weight. The discrepancy between apparent molecular weight and true molecular weight is illustrated in the case of insulin.[2] This highly purified protein was believed to have a molecular weight of from 35,000 to 45,000 by the commonly employed physical methods. Modifications of these procedures suggested at first a molecular weight of 12,000 and then of 6,000. The latter value agrees most closely with the chemical analyses of insulin which indicate one residue of glycine, alanine, and isoleucine per mole of protein.

MOLECULAR WEIGHTS* OF PROTEINS

Protein	*Method*					
	Chemical	*Ultracentrifuge*	*Osmotic Pressure*	*Diffusion*	*Viscosity*	*X-Ray*
Lactalbumin		17,400				
Cytochrome C	13,000	15,600				
Myoglobin		16,900				
Gliadin		27,900	40,000	27,500		
Hordein		27,500				
Zein	23,000	40,000	39,000			
Concanavalin B		42,000				
Crotoxin		30,000				
Insulin†	36,000	36,000	44,000			36,300
Pepsin	34,200	35,500	36,500	36,000	33,000	39,500
Ovalbumin	40,000	41,000	44,300	49,000	37,000	37,500
β-Lactoglobulin	33,400	41,500	35,800	35,400	40,000	34,000
Serum albumin	73,000	64,500	72,400	71,000	70,000	82,000
Hemoglobin (horse)	66,700	65,500	67,000	63,000		67,900
Serum globulin, γ	164,000	153,000	167,000			
Catalase		250,000				
Edestin	53,000	300,000	49,000			
Urease		480,000				
Nucleohistone, calf		2,000,000				
Tobacco mosaic virus		25,000,000				

* Many values are averages from the literature.
† See discussion in text.

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[2] *Nature*, 170, 518 (1952).

Nature of Protein Solutions. Studies of the behavior of the serum globulins led Sørensen, some years ago, to conclude that euglobulin and pseudoglobulin were reversibly combined in a loose chemical combination, E_pP_q, in which E and P represent euglobulin and pseudoglobulin complexes, respectively, combined in the relative proportions of p and q. Subjecting serum to such procedures as dialysis, or fractionation with ammonium sulfate, results in a shifting of the proportions of E and P with the resultant formation of more soluble and less soluble complexes of the two proteins. Sørensen failed, even after repeated fractionations, to prepare samples of either euglobulin or pseudoglobulin that were completely free from the other protein. These results with the globulins led to a series of investigations of other proteins with the result that highly purified preparations of serum albumin, casein, and gliadin were each found to consist of mixtures of an unknown number of proteins of similar character combined in a reversible manner. Such proteins, according to Sørensen, represent "reversibly dissociable component systems" and may be represented by the formula $A_xB_yC_z$ . . . , in which A, B, C, etc., represent components of a definite character and composition (e.g., polypeptides) while x, y, z, etc., indicate the number of such components in the more complex system. In each component, the atoms or groups of atoms, such as amino acids, are linked together by means of strong chemical bonds, whereas the complexes are formed by the union of these components through weak, residual valences. Chemical or physical agents that act on the chemical bonds produce irreversible changes in the protein molecule whereas the residual valences respond to changes in salt concentration, pH, or temperature in a reversible manner. Although the various fractions obtained by the fractionation methods employed possess the essential properties of the initial material, they exhibit variations in physical properties and chemical composition that are considered to be due to the varying amounts of the individual components in each fraction. In no case has Sørensen succeeded in isolating a component which could not be further fractionated by appropriate methods.

Applying these conceptions to biological systems, we find that not only may comparatively simple components combine with each other by means of their residual valences to form protein complexes, but these complexes may themselves combine to form still more complicated structures. In serum, for example, we probably have not only such component systems as albumins and globulins, which may be isolated by suitable methods, but also more complex systems in which these proteins are combined in varying proportions not only with each other but with other serum constituents, such as the lipides. In protoplasm, instead of relatively inert, independent substances, there are probably complex systems composed of protein, lipide, and carbohydrate in equilibrium with each other (*orosins*) and constantly shifting in response to changes in environment. The multiplicity and flexibility of such systems may be of profound importance in determining the adaptability of the organism to its environment.

If we accept Sørensen's views on proteins, we can understand the extraordinary difficulties encountered in the isolation of individual proteins from such complex materials as serum and egg white, and can explain results obtained in Svedberg's laboratory on these substances. Svedberg found, for example, that *there was no substance in fresh egg white with a molecular weight corresponding to that of the crystallized ovalbumin*, but that this substance appeared only after the egg white was treated with ammonium sulfate, as in the crystallization method employed. Similarly, Svedberg found that half-saturation of serum with ammonium sulfate precipitated a globulin fraction which was homogeneous and had a definite molecular weight, and that the euglobulin and pseudoglobulin appeared only after this substance was subjected to further fractionation processes. It thus appears that the proteins with which we are familiar exist in nature only as part of more complex systems, and that even these purified proteins may themselves be complexes formed by the union of several simpler components. The problem of determining the structure and properties of the protein molecule thus depends upon the development of suitable methods for the isolation, in pure form, of the comparatively simple components.

The complexity of the serum proteins is illustrated by the large number of components which one is able to obtain by suitable fractional precipitation of serum. This subject is discussed in detail in Chapter 22.

Colloidal Behavior of Protein Solutions. The proteins form colloidal solutions of the type known as emulsoids, or hydrophilic colloids. One of the most characteristic properties of emulsoids, as pointed out in Chapter 1, is that such systems have two stability factors, charge and hydration, either of which is capable of keeping the particle in solution. Individual proteins show marked differences in the hydration of their particles. Most proteins are soluble in dilute acids and alkalies, the particles acquiring positive or negative charges depending upon the pH of the solution. For every protein, there is a definite characteristic pH, known as the *isoelectric point*, at which the particles are electrically neutral and cease to migrate to the poles of an electrical field. Although all proteins are least soluble at their isoelectric points, certain proteins such as gelatin or ovalbumin remain in solution when brought to their respective isoelectric points. On the other hand, less soluble proteins such as casein and edestin remain in solution only at acid or alkaline reaction and precipitate when their solutions are brought to the isoelectric point. Ovalbumin thus behaves as a typical emulsoid, forming stable solutions of neutral particles. Casein acts more like a suspensoid, the particles of which flocculate when their charges are neutralized. The effect of hydration and charge on the colloidal properties and stability of protein solutions is illustrated in Fig. 56, adapted from Kruyt.

The determination of the true nature of protein solutions is complicated by the fact that the proteins are unique colloids, since in addition to their typically colloidal properties they function as amphoteric electrolytes because of the free amino and carboxyl groups which their molecules contain. A great deal of confusion existed as to whether the reactions of proteins with acids and bases are chemical reactions, taking place in stoichiometric proportions, or colloidal reactions following various laws of adsorption. Much of this confusion is probably due to differences in the nature of the particles present in different protein solutions. The work of Pauli, of Sørensen and of Loeb, and more especially the fine work of Svedberg, discussed above, indicates that in solutions of such proteins as ovalbumin, hemoglobin, and edestin, the material is dispersed in the form of individual molecules of protein and not as molecular aggregates. Such proteins therefore form molecular solutions whose colloidal properties are due entirely to the comparatively large size of the individual molecules. Solutions of other proteins, such as gelatin and casein, probably contain molecular aggregates as well as individual molecules. This point of view is supported by the growing mass of evidence obtained from ultracentrifugal and ultrafiltration experiments as well as from numerous studies on the physicochemical properties of protein solutions.

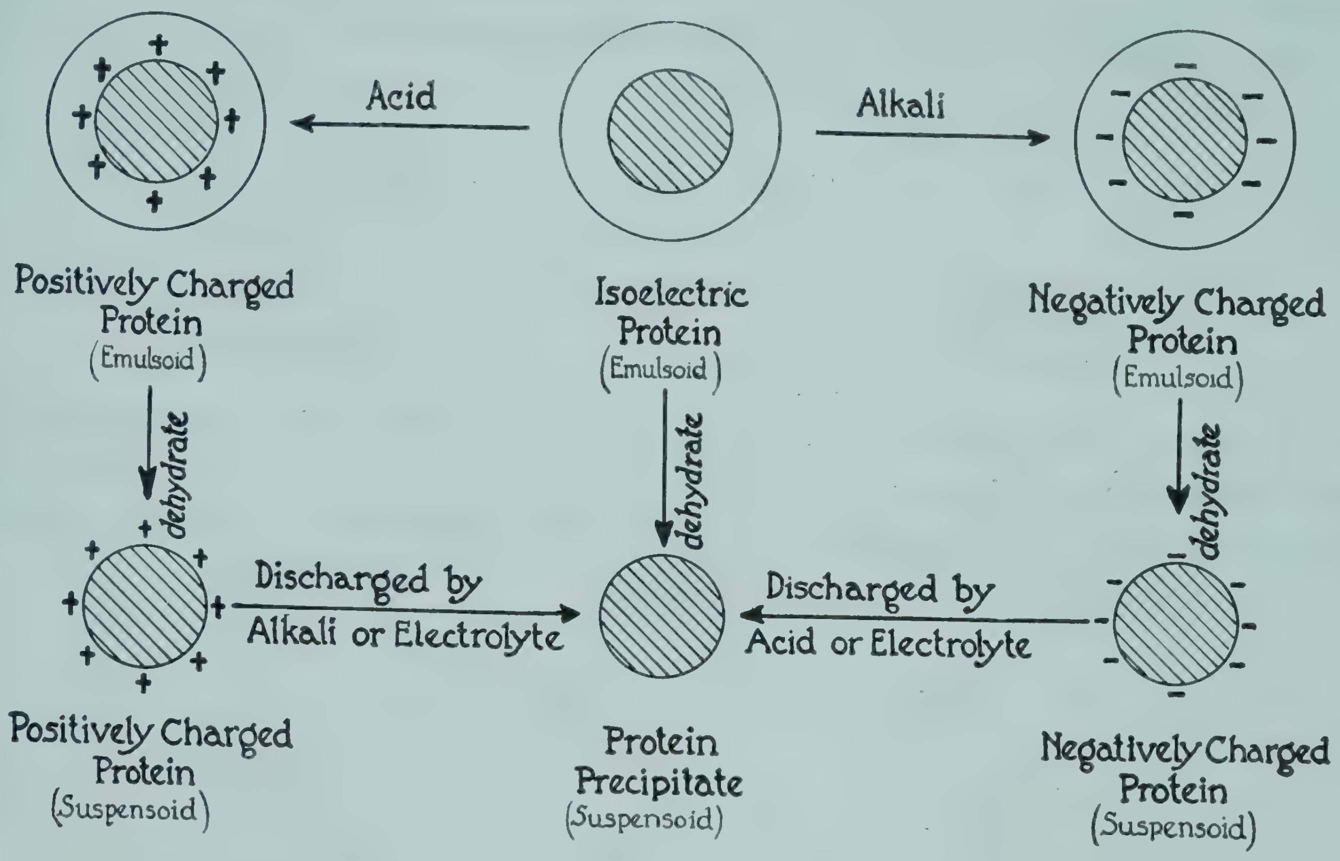
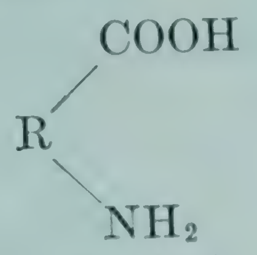

Acid
Alkali
Positively Charged Protein (Emulsoid)
Isoelectric Protein (Emulsoid)
Negatively Charged Protein (Emulsoid)
dehydrate
dehydrate
dehydrate
Discharged by Alkali or Electrolyte
Discharged by Acid or Electrolyte
Positively Charged Protein (Suspensoid)
Protein Precipitate (Suspensoid)
Negatively Charged Protein (Suspensoid)

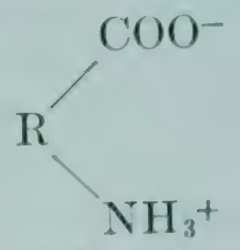
FIG. 56. COLLOIDAL BEHAVIOR OF PROTEIN SOLUTIONS.

Behavior of Proteins as Amphoteric Electrolytes: Isoelectric Points of Proteins. It is currently believed that proteins behave as molecular solutions of amphoteric electrolytes which exhibit typical colloidal properties because of the large size of the individual molecules. These large molecules contain reactive amino and carboxyl groups which are capable of entering into true chemical combination with acids and bases. In choosing between the chemical and colloidal interpretations of protein reactions, it is well to consider, as pointed out by Svedberg, that colloidal and chemical forces are fundamentally the same, since both are electrical in character and depend upon the attraction and repulsion of negative electrons and positive nuclei. It is quite probable that different proteins vary in their reactions because of the fact that solutions of certain proteins undoubtedly contain individual molecules, but others contain molecular aggregates of various sizes. In the present state of our knowledge of protein chemistry we may assume that the proteins exhibit both chemical and colloidal properties, the former being due to the presence of reactive groups in the protein molecule and the latter being dependent upon changes in the charge and hydration of the particles in solution, whether molecules or molecular aggregates.

Considered as amphoteric electrolytes, the proteins combine with acids and bases to form salts which ionize into a colloidal protein ion and one or more crystalloidal ions. According to the work of Loeb, Sørensen, Pauli, Michaelis, Cannan, and their co-workers, this combination takes place in definite proportions in accordance with the laws of classical chemistry. A protein at its isoelectric point is either entirely without charge—i.e., completely un-ionized—or else, according to the *zwitterion* hypothesis of Bjerrum, carries equal numbers of positive and negative charges due to complete dissociation of equal numbers of acid and basic groups in the molecule. Isoelectric protein may thus be represented by the formulas:

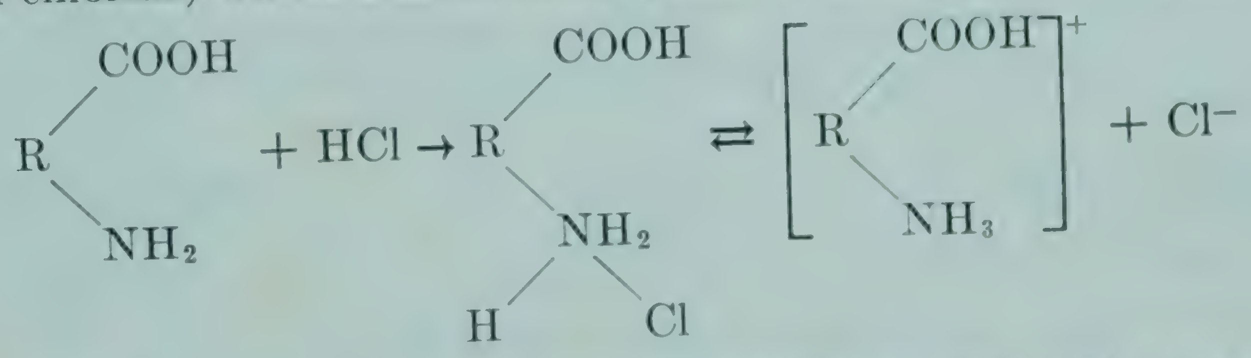


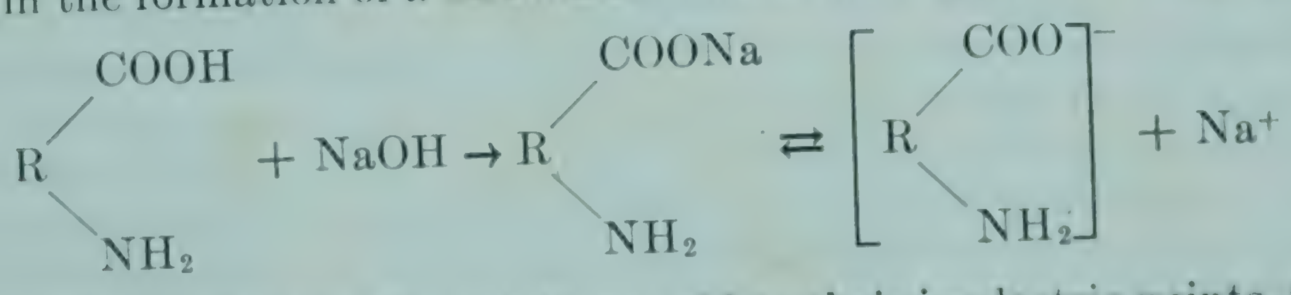
Classical



Zwitterion

Here R stands for the nucleus of the protein molecule. The addition of acid to isoelectric protein results in the formation of a protein salt (e.g., protein chloride) which ionizes into a positive protein ion and a negative acid ion. In the same way the addition of alkali to isoelectric protein results in the formation of a salt which ionizes into a positive metal ion and a negative protein ion.[3] In solutions acid to their isoelectric points, therefore, the proteins exist as positively charged ions, capable of combining with negative ions to form salts, while in solutions alkaline to their isoelectric points proteins exist as negatively charged ions which can combine only with positive ions. Loeb added solutions of silver nitrate and potassium ferrocyanide to powdered gelatin which had been previously brought to various hydrogen-ion concentrations by soaking in appropriate solutions of acid and alkali. He showed that gelatin combines with silver only when the solutions are on the alkaline side of its isoelectric





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[3] The above reactions make use of the older formula for isoelectric protein. The same products are formed according to the zwitterion hypothesis, the only difference being in the mechanism involved. (See section on amino acids, Chapter 4.)

point ($pH > 4.7$), and with ferrocyanide only on the acid side of its isoelectric point ($pH < 4.7$). At the isoelectric point gelatin behaves as though it were un-ionized, since it does not combine with either positive ions (cations) or negative ions (anions).

The isoelectric points of the proteins are of especial significance in protein chemistry, because the properties of the proteins undergo unique changes at these points. It has already been pointed out that proteins are ionized and can enter into chemical combination only in solutions which are acid or alkaline with respect to their isoelectric points. The solubility of the proteins, especially those proteins which resemble the suspensoids in their colloidal behavior, is either negligible or at a minimum at the isoelectric points. Other physical properties of the proteins such as viscosity, osmotic pressure, swelling, etc., are also at a minimum at the isoelectric points. According to Loeb these properties are dependent upon a Donnan equilibrium set up between the particles and the surrounding dispersion medium. The isoelectric points of a number of the more common proteins are given in the following table.

ISOELECTRIC POINTS OF SOME COMMON PROTEINS

Protein	*pH*
Ovalbumin	4.55–4.90
Edestin	5.5–6.0
Serum albumin	4.88
Serum globulin (horse)	5.5
Gelatin	4.80–4.85
Casein	4.55
Hemoglobin (reduced)	6.79–6.83
Hemoglobin (oxidized)	6.7
Gliadin	6.5
Protamines	12.0–12.4
Silk fibroin	2.0–2.4
Myosin	6.2–6.6
Pepsin	<1.0
β-Lactoglobulin	5.2
Insulin	5.30–5.35

EXPERIMENTS ON PROTEINS

The proteins are characterized by the fact that they contain nitrogen, in addition to carbon, hydrogen, and oxygen; by the colloidal nature of their solutions; and by a series of color and precipitation reactions. In the following sections the technique and interpretation of these reactions will be discussed.

A. COMPOSITION TESTS

Since the proteins always contain nitrogen, a positive test for this element indicates the possibility that the material under examination is a protein. A negative test for nitrogen definitely eliminates the possibility of protein.

1. *General Composition Test.* Heat some powdered egg albumin in a dry test tube in which is suspended a strip of moistened red litmus paper and across the mouth of which is placed a piece of filter paper moistened with lead acetate solution. As the powder is heated it chars, indicating the presence of carbon; the fumes of ammonia are evolved, turning the red litmus paper blue and indicating the presence of nitrogen and hydrogen; the lead acetate paper is blackened, indicating the presence of sulfur; and the deposition of moisture on the side of the tube indicates the presence of hydrogen. (Moisture indicates hydrogen only in case both the powder and the test tube are absolutely dry.)

2. *Test for Organic Nitrogen—Lassaigne Test.* Support a clean, dry test tube in a vertical position by means of a clamp placed near the open end. Drop in a small piece of freshly cut sodium (about a 3-mm. cube) which has been wiped free from oil with a piece of filter paper. Heat the bottom of the tube until a layer of sodium vapor about 1 cm. thick is formed. Drop a small amount of the material to be tested (enough to cover the end of a penknife or small spatula) directly on top of the hot sodium, avoiding contact with the upper wall of the tube. Heat strongly until most of the sodium has vaporized or until thick fumes cease to come off. When cool add a few milliliters of distilled water. Heat the contents to boiling and filter. If the fusion with sodium has been carried out satisfactorily, the filtrate will be colorless. Otherwise it should be rejected and the fusion repeated.

Boil 2 ml. of the clear, colorless filtrate for about a minute with a few drops of 10 per cent sodium hydroxide and 1 or 2 (no more) drops of a freshly prepared, saturated solution of ferrous sulfate. Cool, add dilute HCl, drop by drop, until the solution becomes acid and the precipitate of ferrous hydroxide dissolves. The formation of a precipitate of prussian blue (ferric ferrocyanide) or of a blue or green color in the solution indicates the presence of organic nitrogen in the original material. If the solution remains colorless add a drop or two of ferric chloride solution. If nitrogen is present the solution will turn blue or green and a blue precipitate will usually form on standing.

The fusion of a nitrogen-containing organic compound with sodium results in the formation of sodium cyanide. This, when heated with ferrous sulfate in alkaline solution forms sodium ferrocyanide. The sodium ferrocyanide reacts with the ferric ion usually present, or with the added ferric ion, to form the blue ferric ferrocyanide. An excess of acid is to be avoided since the formation of the ferric ferrocyanide is much more delicate in the presence of only a slight excess of acid. The reactions involved are as follows:

$$\begin{aligned} \mathrm{Na + C + N} &= \mathrm{NaCN} \\ \mathrm{2NaCN + Fe(OH)_2} &= \mathrm{Fe(CN)_2 + 2NaOH} \\ \mathrm{4NaCN + Fe(CN)_2} &= \mathrm{Na_4Fe(CN)_6} \\ \mathrm{3Na_4Fe(CN)_6 + 4FeCl_3} &= \mathrm{Fe_4[Fe(CN)_6]_3 + 12NaCl} \end{aligned}$$

An excess of ferrous sulfate must be avoided since, as these equations show, this would tend to stop the reaction at the $Fe(CN)_2$ stage and thus prevent the formation of the characteristic blue ferrocyanide.

3. *Tests for Sulfur.* A. TESTS FOR CYSTINE AND CYSTEINE SULFUR. (1) To equal volumes of KOH and egg albumin solutions in a test tube add 1 to 2 drops of lead acetate solution and boil the mixture. Cystine or cysteine sulfur is indicated by a darkening of the solution, the color deepening into a black if sufficient sulfur is present. Add hydrochloric acid and note the character-

istic odor evolved from the solution. Write the reactions for this test. (2) Place equal volumes of KOH and egg-albumin solutions in a test tube and boil the mixture vigorously. Cool, make acid with glacial acetic acid, and add 1 to 2 drops of lead acetate. A darkening indicates the presence of cysteine or cystine sulfur.

B. TEST FOR TOTAL SULFUR. (*Cystine, Cysteine, and Methionine*). **Place the substance to be examined (powdered egg albumin) in a small porcelain crucible, add a suitable amount of solid fusion mixture (sodium carbonate and potassium nitrate mixed in the proportion 2:1), and heat carefully until a colorless mixture results. (Sodium peroxide may be used in place of this fusion mixture if desired.) Cool, dissolve the cake in a little warm water, and filter. Acidify the filtrate with hydrochloric acid, heat it to the boiling point, and add a small amount of barium chloride solution. A white precipitate forms if sulfur is present. What is this precipitate?**

As mentioned in the preceding chapter, sulfur is present in proteins as cystine, cysteine, or methionine. Cystine and cysteine sulfur was formerly termed *unoxidized, loosely combined, mercaptan*, or *lead-blackening sulfur*. Methionine sulfur because of its greater stability toward alkaline lead acetate was called *oxidized* or *acid sulfur*. These terms are generally misleading and, in the case of the term *oxidized*, incorrect. The majority of proteins contain more methionine S than cystine + cysteine S. Exceptions are keratins, insulin, and certain serum albumins which contain all or almost all their sulfur in the form of cystine and cysteine. Silk fibroin and many protamines are devoid of sulfur.

B. COLOR REACTIONS OF PROTEINS

These color reactions are due to a reaction between some one or more of the constituent radicals or groups of the complex protein molecule and the chemical reagent or reagents used in any given test. Not all proteins contain the same amino acids and for this reason the various color tests will yield reactions varying in intensity of color according to the nature and amount of the groups contained in the particular protein under examination. Various substances not proteins respond to certain of these color reactions, and it is therefore essential to submit the material under examination to several tests before concluding definitely regarding its nature.

1. Millon's Reaction. **To 5 ml. of a dilute solution of egg albumin[4] in a test tube, add 3 to 4 drops of Millon's reagent.[5] Mix and bring the mixture gradually to the boiling point by heating over a small flame. Proteins like egg albumin, which are precipitated by strong mineral acids, yield a white precipitate which gradually turns red upon heating; whereas other proteins, like the secondary proteoses and peptones, yield only a red solution under the same conditions. If no color develops, add 2 to 3 more drops of the reagent**

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[4] This egg albumin solution may be prepared by beating egg white with 6 to 10 volumes of water. The material is strained through cheesecloth to remove the precipitate of ovomucin formed, and then filtered through filter paper and the filtrate used in the tests. A 1 per cent solution may also be prepared from powdered or scale egg albumin by soaking the material in a small quantity of water (sufficient to moisten it thoroughly) for several hours, then diluting to volume, stirring until dissolved, and filtering.

[5] See Appendix.

and heat again. An excess of the reagent is however to be avoided since it may produce a yellow color which is not a positive reaction. Repeat the test, using a 0.1 per cent solution of phenol instead of the protein, and note the red color produced upon heating.

This test is a particularly satisfactory one for use on solid proteins. In this case, dilute some of the reagent with 3 to 4 volumes of distilled water, add the solid, and heat gently as above. The particles of undissolved protein will gradually turn red; if any of the protein dissolves, the solution will also assume a red color.

The reaction is due to the presence of the hydroxyphenyl group, —C_6H_4OH, in the protein molecule; and any phenolic compound which is unsubstituted in the 3,5 positions such as tyrosine, phenol (carbolic acid), and thymol will give the reaction. Inasmuch as the tyrosine or halogenated tyrosine grouping is the only hydroxyphenyl grouping which definitely has been proved to be present in the protein molecule, it is evident that proteins respond to Millon's reaction because of the presence of this amino acid. The test is not a very satisfactory one for use in solutions containing inorganic salts in large amount, since the mercury of the Millon's reagent is thus precipitated and the reagent rendered inert. This reagent is therefore never used for the detection of protein material in the urine. If the solution under examination is strongly alkaline it should be neutralized, inasmuch as the alkali will precipitate yellow or black oxides of mercury.

2. *Millon-Nasse Reaction*. This is an adaptation of the Millon test and can be used in the presence of considerable quantities of inorganic salts, especially NaCl.

To 5 ml. of a dilute solution of protein in a test tube, add 1 ml. of a 15 per cent solution of mercuric sulfate in 6 N sulfuric acid. Place the tube in a boiling water bath for 10 minutes, cool the contents in water for 5 to 10 minutes, and add 1 ml. of 1 per cent $NaNO_2$. A deep-red color indicates tyrosine or other 3,5 unsubstituted phenol.

3. *Xanthoproteic Reaction*. To 2 to 3 ml. of egg-albumin solution in a test tube add 1 ml. of concentrated nitric acid. A white precipitate forms, and upon heating turns yellow and finally dissolves, imparting to the solution a yellow color. Cool the solution and carefully add ammonium hydroxide or sodium hydroxide in excess. Note that the yellow color deepens into an orange. Repeat the test using a 0.1 per cent phenol solution instead of the protein, and note the production of the yellow and, later, the orange color.

This reaction is due to the presence in the protein molecule of the phenyl group, —C_6H_5, with which the nitric acid forms certain nitro modifications. The particular complexes of the protein molecule which are of especial importance in this connection are those of tyrosine and tryptophan. Phenylalanine does not respond to this test as it is ordinarily performed. The test is not a satisfactory one for use in urinary examination because of the color of the end reaction.

4. *Glyoxylic Acid Reaction* (*Hopkins-Cole*). Place 2 to 3 ml. of egg albumin solution and an equal volume of glyoxylic acid ($CHO{\cdot}COOH + H_2O$ or $CH(OH)_2COOH$) solution (Hopkins-Cole reagent)[6] in a test tube and mix thoroughly. Incline the tube and permit 5 to 6 ml. of concentrated sulfuric acid to flow slowly down the side of the tube, thus forming a sharp layer of acid

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[6] See Appendix.

beneath the protein mixture. When stratified in this manner a reddish-violet color forms at the zone of contact of the two fluids. If the color does not appear after standing for a few minutes, the tube may be rocked gently to cause a slight mixing of the liquids at the interface. If the two liquids are mixed by gentle stirring the precipitate of protein dissolves and the violet color spreads throughout the solution.

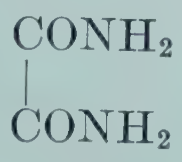
This color is due to the presence of the *tryptophan group* (see p. 142). Gelatin does not respond to this test. Nitrates ($NaNO_3$ and KNO_3), chlorates, nitrites, or excess of chlorides prevent the reaction, but a trace of copper sulfate will increase its sensitivity. The sulfuric acid used must be pure.

5. *Biuret Test*. To 2 to 3 ml. of egg-albumin solution in a test tube add an equal volume of 10 per cent sodium hydroxide solution, mix thoroughly, and add a 0.5 per cent copper sulfate solution drop by drop, mixing between drops, until a purplish-violet or pinkish-violet color is produced. (If too much copper sulfate is added the violet color may be obscured by the blue precipitate of copper hydroxide formed.) The color depends upon the nature of the protein: proteoses and peptones give a decided pink; the color produced with gelatin is not far removed from a blue.

Repeat the biuret test on some biuret, formed from urea as follows: Place about one-eighth inch of urea in a clean dry test tube and heat gently over a small flame. The urea melts and then effervesces, and the biuret formed appears as a white solid in the bottom of the tube. Note the odor of the gas given off during the heating. Allow the tube to cool, dissolve the biuret in 3 to 4 ml. of 10 per cent sodium hydroxide, and add 0.5 per cent copper sulfate solution, drop by drop, until the pink color appears.

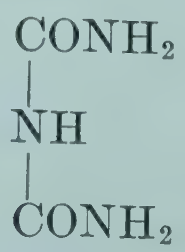
The biuret test may also be carried out with a stable biuret reagent, prepared by adding 1 per cent copper sulfate solution, drop by drop, with constant stirring, to some 40 per cent sodium hydroxide solution until the mixture assumes a deep blue color. This reagent may be used in either of two ways. It may be added directly to the protein solution, a drop at a time, with mixing, until the solution assumes a violet color; or two or three drops of the reagent may be permitted to flow down the side of the inclined tube. In this case the reagent forms a layer beneath the protein solution and the violet color appears at the interface between the two liquids.

The biuret test is given by those substances whose molecules contain two carbamyl (—$CONH_2$) groups joined either directly together or through a single atom of nitrogen or carbon. Similar substances which contain (in place of the —$CONH_2$ group) —$CSNH_2$, —$C(NH)NH_2$, or —CH_2NH_2 also respond to the test. It follows from this fact that substances which are nonprotein in character but which contain the necessary groups will respond to the biuret test. As examples of such substances the following may be cited:



$$\begin{array}{l} CONH_2 \\ | \\ CONH_2 \end{array}$$

Oxamide

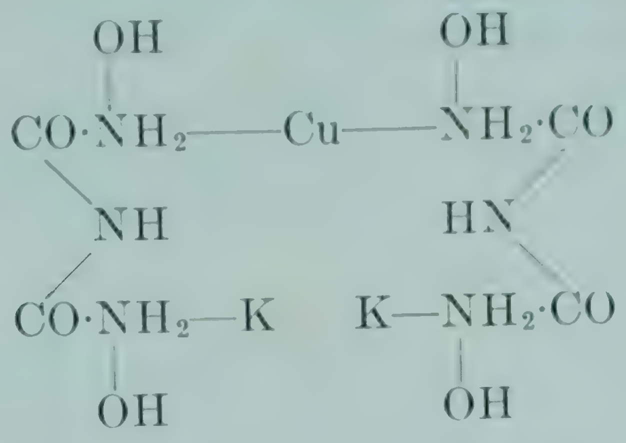


$$\begin{array}{l} CONH_2 \\ | \\ NH \\ | \\ CONH_2 \end{array}$$

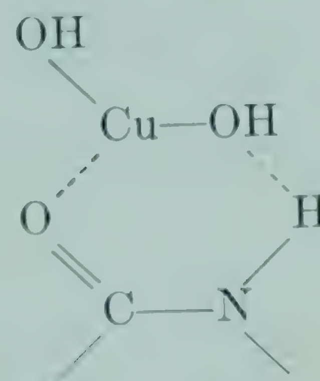
Biuret

The test derives its name from the fact that biuret, which is formed on heating urea to 180° C., responds to the test. Proteins respond positively since there are pairs of CONH groups in the molecule.

According to Schiff the end reaction of the biuret test is dependent upon the formation of a copper-potassium-biuret compound (cupripotassium biuret or biuret potassium cupric hydroxide). This substance was obtained by Schiff in the form of long red needles and has been formulated as follows:



Haurowitz[7] assumes that a copper coordination complex with the following ring structure is probably produced:



If much magnesium sulfate is present a precipitate of magnesium hydroxide forms which interferes with the test. If much ammonium sulfate is present a large excess of alkali must be used.

6. *The Triketohydrindene Hydrate (Ninhydrin) Reaction.* To 5 ml. of dilute protein solution, which must be approximately between pH 5 and pH 7,[8] add 0.5 ml. of a 0.1 per cent solution of triketohydrindene hydrate, heat to boiling for one to two minutes, and allow to cool. A blue color develops if the test is positive.

This test gives positive results with proteins, peptones, peptides, amino acids, and other primary amines, including ammonia. For further discussion see p. 129.

C. PRECIPITATION REACTIONS OF PROTEINS

The proteins are precipitated from solution by salts of the heavy metals (e.g., $HgCl_2$, $AgNO_3$, $CuSO_4$, $Pb(C_2H_3O_2)_2$, etc.); by certain acids some of which are called *alkaloidal reagents* (picric acid, phosphotungstic acid, tannic acid, metaphosphoric acid, etc.); by concentrated solutions of such salts as ammonium sulfate, sodium sulfate, and sodium chloride; and by ethyl and methyl alcohol. Although these reactions have been used for many years for the separation and characterization of proteins, there is still no definite evidence concerning the nature of the mechanisms involved. Some of this uncertainty is due to the experimental difficulties involved in the isolation of the pure products formed in these reactions. There is also a great deal of confusion due to attempts by various authors to interpret these reactions exclusively on either a chemical or a colloidal basis.

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[7] Haurowitz: *Chemistry and Biology of Proteins*, New York, Academic Press, Inc., 1950, p. 11.

[8] A few drops of pyridine or a few crystals of sodium acetate may be used to adjust the pH.

In the case of the acids, the evidence suggests that the protein combines with the acid radical to form insoluble salts, such as protein tannate, protein phosphotungstate, etc. This is in agreement with the views of Loeb, who pointed out that proteins combine with negative, or acid, ions only on the acid side of their isoelectric points. In agreement also is the fact that if, instead of the free acids, the salts of these acids are added to neutral solutions of protein, no precipitates result until the solution is acidified. Some of the reactions obtained with the salts of the heavy metals are probably similar in character; precipitates of silver proteinate, for example, are formed on the alkaline side of the isoelectric points. The reactions are, however, complicated by the fact that in some cases the first addition of salt causes the formation of a precipitate which dissolves in excess of salt and then reprecipitates when more salt is added. The difficulties involved in arriving at a rational explanation of these reactions are intensified by the fact that individual proteins show great differences in behavior, probably because of differences in the hydration and dispersion of the particles in solution, denaturation, etc. In general, precipitants of this class usually cause extensive intramolecular changes in the protein molecule.

The proteins, in common with other emulsoids, are precipitated by concentrated solutions of such salts as ammonium sulfate, sodium sulfate, sodium chloride, etc. In these cases, as discussed in Chapter 1, precipitation is apparently due to the neutralization and dehydration of the molecules and molecular aggregates in solution. The protein precipitated by these salting-out methods is unaltered (native) and usually redissolves when treated with fresh portions of the original solvent. The concentration of salt required for the precipitation of a protein depends on the particular protein and on the pH of the solution—i.e., on the charge on the protein complex. These variations will be considered in the next chapter in connection with the properties of the various classes of proteins.

The proteins are also precipitated by dehydrating agents, such as alcohols and acetone. The addition of alcohol to electrolyte-free solutions of proteins converts them into suspensoids, which flocculate upon the addition of a few drops of salt solution. Precipitation by alcohol is most effective at the isoelectric point of the protein. Besides precipitating proteins, alcohol acts on certain proteins, such as egg albumin, to produce intramolecular changes which affect the solubility and other properties of the protein. This phenomenon, known as denaturation, will be discussed more fully in the next section. The denaturing effect of alcohol on some proteins may be minimized by the use of temperatures around 0° C. or below. Prolonged contact with alcohol (or any other denaturing agent, for that matter) produces an irreversible coagulation of the protein. The fixing of tissues for histological examination is an example of the coagulating action of alcohol on proteins.

Colloidal iron, kaolin, and alumina cream are frequently used for removing proteins from solution. These substances probably act by adsorption and their use has been adapted to various quantitative methods.

1. ***Effect of Strong Acid and Alkali.*** Place a few ml. of concentrated nitric acid in a test tube, incline the tube, and add dilute egg albumin slowly from a pipet, allowing the solution to run down the side of the tube and form a layer over the nitric acid. Note the appearance of a protein precipitate at the zone of contact between the two fluids. Now mix the contents of the tube thoroughly by careful shaking. Is protein precipitated by concentrated nitric acid?

Repeat the above experiment using concentrated sulfuric acid, concentrated hydrochloric acid, acetic acid, and concentrated sodium hydroxide. How do these various reagents differ in their action on proteins? Allow the tubes to stand overnight or longer and note any further changes.

The formation of a protein precipitate by layering the solution over nitric acid as described above is frequently used as a test for protein in urine and other fluids (Heller's test, see p. 830).

2. ***Precipitation by Metallic Salts.*** Prepare six tubes each containing 2 to 3 ml. of dilute egg-albumin solution. To the first add mercuric chloride solution, drop by drop slowly, until an excess of the reagent has been added, and note any changes which may occur. Unless the reagent is added very gradually the formation of the precipitate may not be noted, due to its solubility in excess of the reagent. Repeat the experiment with lead acetate, silver nitrate, copper sulfate, ferric chloride, and barium chloride, using very dilute solutions.

Egg albumin is used as an antidote for lead or mercury poisoning. Why? Is it an equally good antidote for the other metallic salts tested?

3. ***Precipitation by Alkaloidal Reagents.*** Prepare six tubes, each containing 2 to 3 ml. of dilute egg-albumin solution. To the first add picric acid, drop by drop, until an excess of the reagent has been added, noting any changes which may occur. Repeat the experiment with trichloroacetic acid, tannic acid, phosphotungstic acid, phosphomolybdic acid, and potassio-mercuric iodide. Are these precipitates soluble in excess of the reagent? Acidify with hydrochloric acid before testing with the last three reagents.

4. ***Precipitation by Ferrocyanide.*** To 5 ml. of dilute egg-albumin solution in a test tube add 5 to 10 drops of acetic acid. Mix well and add potassium ferrocyanide, drop by drop, until a precipitate forms. This reaction is very sensitive.

5. ***Fractional Precipitation of Proteins by Concentrated Salt Solution.*** (a) Obtain some dilute egg-white solution which has been prepared by thoroughly mixing one volume of raw egg white with four volumes of 1 per cent sodium chloride solution and filtering. To a portion of the dilute egg-white solution add an equal volume of a saturated solution of ammonium sulfate and mix. Does egg white contain a protein which is precipitated by half-saturated ammonium sulfate solution? Dilute a portion of the mixture with some distilled water. Is the precipitation reversible? To the remainder of the mixture add an excess of solid ammonium sulfate and stir until the

solution is saturated with the salt. What happens? Again dilute a portion of the mixture with water. Is this precipitation reversible? Filter the remainder of the mixture and test a portion of the precipitate by the Millon test. Test the filtrate by the biuret test, using a saturated solution of ammonium sulfate as a control and adding the same amounts of alkali and copper sulfate solution to both control and filtrate. What are your conclusions?

(b) Repeat the above experiment, using sodium chloride instead of ammonium sulfate. How do the results differ from those obtained with ammonium sulfate? At the saturation point with sodium chloride, add 2 to 3 drops of acetic acid. What occurs?

All proteins except peptones are precipitated by saturating their solutions with ammonium sulfate. Most globulins are precipitated by half-saturation with ammonium sulfate or full saturation with sodium chloride. If the saturated sodium chloride solution is subsequently acidified, all proteins except peptones are precipitated.

Soaps may be salted out in a similar manner (see p. 108).

6. Precipitation by Alcohol. (a) INFLUENCE OF ELECTROLYTES. **Prepare an electrolyte-free solution of egg albumin as follows: Place the albumin solution in a dialyzing bag (see p. 10), add a drop of toluene as a preservative, and tie the mouth of the bag securely. Immerse in a large beaker of distilled water and allow to stand for several days, changing the water at intervals. Remove the albumin solution from the bag and filter. Test the filtrate for chloride. It should be negative; if not, the dialysis must be repeated.**

Place a 5-ml. portion of the salt-free albumin solution in each of two test tubes and add 10 ml. of 95 per cent alcohol to each tube. Mix. Now to one tube add a pinch or two of solid sodium chloride and again mix. Compare results in the two tubes. What is the effect of alcohol on protein in the absence of electrolytes? What is the effect of added electrolyte? Explain. Dilute some of the suspension with water. Does the precipitated protein redissolve?

(b) INFLUENCE OF ISOELECTRIC POINT. **See Exp. 2, below.**

D. ISOELECTRIC POINTS OF PROTEINS

1. Isoelectric Point and Solubility of Casein. **Into a 50-ml. volumetric flask introduce 0.25 g. of pure casein. Add about 20 ml. of water and exactly 5 ml. of N NaOH. When solution is complete add exactly 5 ml. of N acetic acid and dilute to 50 ml. Mix well. This is a solution of casein in 0.1 N sodium acetate. Set up a series of nine tubes as follows:**

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Distilled water ml.	8.38	7.75	8.75	8.5	8	7	5	1	7.4
0.01 N acetic acid ml.	0.62	1.25	..	..	..	..	..	..	..
0.1 N acetic acid ml.	..	..	0.25	0.5	1	2	4	8	..
1.0 N acetic acid ml.	..	..	..	..	..	..	..	..	1.6

To each tube add 1 ml. of the casein-sodium acetate solution, blowing it in from a pipet and shaking the tubes immediately. Note the turbidities just after mixing and after 10 and 30 minutes. Record the results as below,

indicating no turbidity by 0, degrees of turbidity by + signs, and degrees of precipitation by ×.

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
pH	5.9	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5
Turbidity, immediate	0	0	+	++	+++	++	+	+	0
Turbidity after 5 minutes	0	0	+	+++	×××	××	++	+	0

The precipitation should be greatest in Tube 5 which has a pH of 4.7, near the isoelectric point and point of least solubility of casein. The acidity in each tube may be actually determined by the electrometric method or may be calculated from the concentrations of sodium acetate and acetic acid by means of the Henderson-Hasselbalch equation (see p. 33).

2. *Isoelectric Point and Precipitation of Gelatin by Alcohol.* Gelatin and many other proteins are quite soluble in water even at their isoelectric points. They do, however, precipitate more readily at this point if some precipitating agent such as alcohol is added.

Prepare a series of test tubes as follows:

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
0.1 N sodium acetate ml	2.00	2.00	2.0	2	2	2	2.0	2.0	2.0
0.1 N acetic acid ml	0.12	0.25	0.5	1	2	4	. .	. .	. .
1.0 N acetic acid ml	. .	. .	. .	. .	. .	. .	0.8	1.6	3.2
Distilled water ml	3.88	3.75	3.5	3	2	0	3.2	2.4	0.8
1% gelatin ml	2.00	2.00	2.0	2	2	2	2.0	2.0	2.0

Mix the contents of the tubes well and add 95 per cent alcohol to Tube 5 until a very faint cloudiness is produced (ordinarily about 8 ml. are required). Add the same amount of alcohol to each of the other tubes. Examine after 30 minutes. The results usually obtained, with approximate pH for each tube, are as follows:

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Turbidity	–	–	–	++	+++	±	–	–	–
pH	6.0	5.6	5.3	5.0	4.7	4.4	4.1	3.8	3.5

The isoelectric point for gelatin is about pH 4.7.

3. *Influence of Acid on Swelling of Gelatin.* Proteins are least hydrated at their isoelectric points. The addition of acid, such as HCl, to gelatin causes it to swell, the process being one of increased hydration. Ions have a strong affinity for water and the addition of acid increases the ionization of the gelatin. In the case of HCl the H ion tends to increase the swelling and the Cl ion to depress it. In high concentrations the latter effect overcomes the former and decreased swelling results. The same result follows the addition of Cl ion as NaCl, which hence tends to decrease swelling. The influence of ions and other substances on the hydration of cell proteins is believed to be

of great biological importance. Relatively slight changes in pH greatly influence protein swelling.

Prepare a series of test tubes as follows:

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
1.0 N HCl ml	16	8	4	..	..	..	..	..	..	..
0.1 N HCl ml	..	..	..	20	10	5	2.5	1.25	0.62	0.31
Distilled water ml	4	12	16	0	10	15	17.5	18.75	19.38	19.69

Cut strips as nearly the same size as possible (5 cm. × 0.5 cm.) from a sheet of gelatin at least 1 mm. thick. Put a strip in each tube. After 24 hours measure the length of the strips. Results similar to the following may be obtained.

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Length (cm.)	6.4	6.6	6.7	7.4	8.3	8.3	7.6	7.0	6.1	5.3

The greatest degree of swelling will usually be found in tubes containing from 0.025 to 0.05 N HCl corresponding to pH values of 1.3 to 1.6. The reaction within the gelatin particles themselves at the point of maximum swelling is, however, about pH 3.2. This difference in reaction within and without the gel is a consequence of the Donnan equilibrium (see p. 12) which must be considered in all such cases involving the relations of protein ions to other ions in solution. Instead of gelatin strips, 1-g. portions of powdered gelatin (between 30 and 50 mesh) may be placed in 100-ml. graduated cylinders containing suitable solutions and the relative degree of swelling measured. The actual pH of the gel in any case may be determined electrometrically.

4. *Influence of Acidity on Solidification of Gelatin.* The isoelectric point for gelatin (pH 4.7) is the point of minimum viscosity of its solutions and the point at which the gelatin most readily solidifies. This is because at this point the gelatin is least soluble in the more liquid (water) phase and hence imparts less viscosity to it. For the same reason there is a greater tendency for the more solid phase (gelatin) to form the supporting network believed to be the basis of a jelly. Viscosity determinations must of course be made on the liquefied gelatin solution.

Prepare a series of test tubes as follows:

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*
0.1 N sodium acetate ml	1.00	1.00	1.00	1.0	1.0	1.0	1.0
0.1 N acetic acid ml	0	0.06	0.25	1.0	4.0	..	..
1.0 N acetic acid ml	..	..	..	..	..	1.6	6.4
1.0 N sodium hydroxide ml	0.05	..	..	..	..	..	..
Water ml	6.95	6.94	6.75	6.0	3.0	5.4	0.6
pH (approx.)	8.00	5.60	5.00	4.6	4.0	3.4	2.8

To each tube add 3 ml. of a warm 10 per cent solution of gelatin and mix. Place all the tubes in water at 50° C. for a few minutes, then transfer to a large beaker of water at room temperature, noting the time at this point. After a

few minutes in the beaker, the tubes may be removed and placed in a rack to facilitate observation. Note the time required for solidification of the gelatin in each tube. Degree of solidification may be established by tilting the tube and observing whether or not the fluid will flow. Results similar to the following should be obtained.

Tube No.	*1*	*2*	*3*	*4*	*5*	*6*	*7*
Time required for solidification (min.)	19	17	16	14	19	25	very long

Observations may be repeated by remelting the jellies. Note also the much greater opacity of the gelatin at the isoelectric point, indicating that most of the gelatin is in the solid phase.

E. PROTEIN DENATURATION AND ITS REVERSAL

A protein is called a native protein if its amino acid composition and stereochemical structure are unchanged from the natural state. These properties control all the functions of a protein, whether solubility in dilute salt solutions, proteolytic activity, oxygen-carrying capacity, or whatever it may be. These characteristics are altered and the process of denaturation is said to occur when a protein undergoes changes in structure or composition. Chemical and physical agents which cause these changes are called denaturing agents. Their action involves the splitting of some or all of the protein cross linkages with their possible re-formation, in some cases, to cause a rearrangement of the peptide chains.

It is to be expected that proteins will vary widely in their ease of denaturation. Certain proteins, especially those which in solution are fibrous or highly elongated molecules, such as the muscle protein myosin, are easily denatured; others, such as the carbohydrate-rich glycoproteins (ovomucoid, for example) seem to be quite resistant to the usual physical agents causing denaturation.

Denaturation may be caused by: (1) heat, which causes splitting of the salt bridges by thermal agitation; (2) mineral acids and alkalies, which alter the ionization of carboxyl and amino groups respectively and thus destroy salt bridges; (3) shaking or stirring, which results in the denaturation of the protein spread over the surface of the air bubbles; (4) grinding, which probably causes mechanical deformation of the peptide chains; (5) ultraviolet radiation, which splits the peptide bonds adjacent to the aromatic rings; (6) ultrasonic waves, which destroy the rings of the aromatic amino acids and whose denaturing power depends on their frequency; (7) neutral chemical agents such as urea and certain acid and guanidine derivatives that act as dipoles to cause cleavage of hydrogen bonds and formation of new ones.[9] It has been suggested[10] that the breaking of hydrogen bonds by urea enables the —SH groups to react with intramolecular —S—S— bridges each of which is then converted into an intermolecular link with the liberation of a new —SH group. The continuation of this chain process gives rise to an —S—S— cross-linked framework of higher molecular weight and decreased solubility at the isoelectric point. This reaction is formulated as follows:

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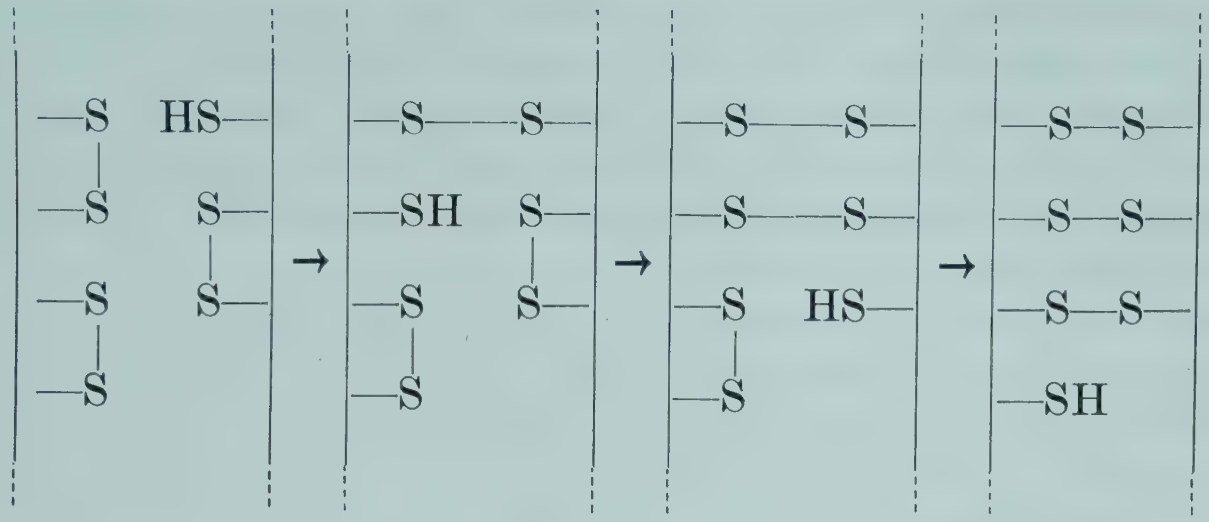
[9] It has been speculated by Pauling that denaturation at times may consist of end to end aggregation of globular protein molecules. *Nature*, **170**, 518 (1952).

[10] Huggins, et al.: *Nature*, **167**, 592 (1951).



Denaturation, then, appears to be an all-or-none phenomenon; that is, a protein is either denatured or unchanged from its natural state. Denatured proteins may, under certain conditions, be restored to proteins with many of the properties of the original protein. This is known as renaturation. It appears, however, that renaturation seldom results in the complete transformation of the denatured protein to its original state. Thus we may classify proteins into three groups: natural, denatured, and renatured.

If the foregoing rather specific definition of denaturation is used, then as already stated protein denaturation must have an all-or-none character. However, denaturation seldom is completed in one step but consists of a series of chemical and structural deviations from the original native protein. If these changes are not too extensive, many of the properties possessed by the native protein may be restored. Thus pepsin can be denatured and so lose its proteolytic properties if it is warmed to the proper temperature. When the solution is cooled, the proteolytic activity of the protein is restored—i.e., denaturation is reversed and renaturation results. Likewise, the oxygen-carrying capacity of hemoglobin can be destroyed by denaturing with salicylate. On reversal the restored hemoglobin is very similar to the original untreated hemoglobin. Although many of the properties of the native protein can be restored by reversal regeneration, it is still questionable in many instances whether the reversed protein is *identical* with the original native protein. Many regenerated proteins may simulate certain characteristic properties of the native materials from which they were derived, but still remain denatured proteins.

It is thus apparent that the extent of denaturation and its reversal is primarily dependent upon the methods used. Probably the oldest means of denaturing a protein and one which is familiar to all is illustrated by the marked change in consistency of egg white on cooking, a process which results in extensive denaturation of the albumen proteins. What is seen when egg white is cooked is only the end result of denaturation—the matting together of the fibers of denatured protein to form a tightly adhering coagulum. Denaturation involving less deep-seated changes in protein structure requires more refined methods to detect. The studies of

Roche, Neurath, and others have shown that denatured proteins which other investigators believed to have been entirely reversed to the native form were still denatured when more delicate methods of measurement were employed.

In practice, then, denaturation consists of a series of changes in the protein molecule brought about by various chemical and physical agents. These changes often affect the viscosity, particle size, solubility, resistance to proteolytic enzymes, and formation or disappearance of sulfhydryl groups, and may even cause the loss of certain amino acids or peptides of relatively low molecular weight.

Denatured proteins, because of their reduced solubility, usually flocculate at or near the isoelectric point. This flocculation is ordinarily reversible at room temperature. However, if the suspension at the isoelectric point is heated, the floccules form relatively large, tenacious masses of *coagulated protein* which are not easily redissolved by treatment with dilute acids or alkalies. Denaturation is the primary and important change; flocculation and coagulation, which were often confused with denaturation, are simply visible manifestations of the changes in the protein molecule brought about by denaturation.

1. *Denaturation, Flocculation, and Coagulation of Egg Albumin.* Place 9 ml. of a clear, salt-free solution of egg albumin into each of three test tubes. To the first add 1 ml. of 0.1 N HCl, to the second 1 ml. of a mixture of sodium acetate and acetic acid (pH 4.7), and to the third 1 ml. of 0.1 N NaOH. Heat all three tubes in a boiling water bath for 15 minutes. Cool and examine. To tubes 1 and 3 add 10 ml. of acetate buffer solution, pH 4.7. What happens? Filter off the precipitates in each tube and wash them on the filter papers with distilled water. Precipitates 1 and 3 are denatured egg albumin. Precipitate 2 is coagulated egg albumin.

Suspend each of the precipitates in about 10 ml. of distilled water and divide each suspension into three parts. To the first part add dilute HCl, drop by drop. Does the precipitate dissolve? Repeat with another part of the suspension, adding dilute NaOH. Heat the third parts of suspensions 1 and 3 in a boiling water bath for 15 minutes. Cool and test the solubility of the material in dilute acid and alkali. Does the material now dissolve?

The precipitates of denatured protein, formed by bringing their solutions to the isoelectric point, dissolve readily in a few drops of dilute acid or alkali. The coagulated protein remains insoluble under similar conditions. Heating the precipitate of denatured protein converts it into a coagulated protein, in which case it exhibits the properties characteristic of that type of substance.

F. IMMUNOLOGICAL REACTIONS OF PROTEINS

Certain materials, when injected into a living animal under proper conditions, stimulate the animal to produce substances which have the power to react with the specific material injected. The substance injected is referred to as the *antigen*, while the specific substances appearing in the blood of the immunized animal are known as *antibodies*. In order to act as antigens the substances must be protein (or combinable with protein) or polysaccharide in nature, colloidal, and foreign to the blood stream of the animal injected. The antibodies are structurally (and possibly chemically) modified serum proteins, usually globulins, induced by the presence of the antigen in those organs in which the formation of serum proteins occurs. Their presence in the blood stream of the immunized animal is recognized by their action on antigens. The type of reaction which takes place between the antigen and antibody depends upon the nature of the antigen. If, for example, the antigen consists of a suspension of red blood corpuscles, the blood serum of the immunized animal, known as the antiserum, acquires the property of hemolyzing this particular type of corpuscle. If the antigen is a suspension of bacteria or other cells, the antiserum will cause these particular cells to clump together or agglutinate.

If the antigen is a soluble protein the antiserum is capable of forming a precipitate with this specific protein, when mixed under proper conditions. This reaction, known as the precipitin reaction, is remarkably delicate and specific. By means of the precipitin reaction it is possible, for example, to detect a specific protein in solutions containing as little as one part in ten million, or even in one hundred million. Furthermore, this reaction is so specific that the antiserum prepared by injecting a solution of hen's egg albumin will precipitate hen's egg albumin but will give no reactions at extreme dilutions with egg albumins from other sources, or with any of the other proteins in egg white. The precipitin reaction is thus of great value in determining the identity and individuality of protein preparations. It permits us to detect differences between similar proteins from closely related sources which, in their chemical composition and properties, are practically indistinguishable. By means of this reaction it may be shown that certain proteins, such as the protein of crystalline lens, are immunologically the same in animals of widely differing species. It has been used to study relationships between various species of plants and of animals. Practically, it is used in medicolegal work to distinguish specifically between human and other bloodstains.

The technique involved in immunization and in carrying out precipitin reactions is illustrated in an experiment on p. 485.

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6

Proteins: Their Classification and Properties

The classification of the proteins is a very difficult problem because, as was outlined in Chapter 5, they are found in nature not as distinct chemical individuals but as parts of larger complexes formed by the union of various proteins with each other and with carbohydrates and lipides. The problem is further complicated by the fact that individual purified proteins may themselves be separated into components showing individual differences in composition and properties. Until these components are isolated in pure form and their exact structure determined, a classification of proteins based on molecular structure is impossible. The classification offered below is based upon the fact that under specified experimental conditions the proteins in biological materials (e.g., egg white or blood plasma) may be separated into groups which exhibit certain characteristic properties. It should be kept in mind, however, that these proteins probably do not exist as such in the native material, and that, furthermore, their composition and properties are dependent upon the methods used in their isolation. In spite of all its shortcomings such a classification permits the grouping together of proteins with similar properties into fairly definite classes, and must suffice until the information required for a more rigid classification is obtained.

CLASSIFICATION

I. SIMPLE PROTEINS

The simple proteins are proteins which may be isolated from biological materials by suitable methods. Although they are usually described as yielding only α-amino acids or their derivatives on hydrolysis, some members of this class (e.g., ovalbumin, serum albumin, serum globulin) also yield carbohydrates (see p. 119). Many proteins, such as egg albumin, pepsin, trypsin, insulin, hemoglobin, serum albumin, edestin, excelsin, viruses, and Bence-Jones and other urinary proteins, may be obtained in crystalline form. By repeated crystallizations these proteins may thus be separated from other proteins present in the original materials. The simple proteins are further classified into albumins, globulins, glutelins, prolamins, albuminoids, histones, globins, and protamines on the basis of solubility and other characteristic properties, as follows.[1]

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[1] The subclasses defined are exemplified by proteins obtained from both plants and animals. Appropriate prefixes indicate the origin of the compounds, e.g., ovoglobulin, **lactalbumin, etc.**

Albumins are soluble in salt-free water and coagulable by heat, e.g., ovalbumin from egg white, serum albumin from blood serum, lactalbumins from milk, vegetable albumins.

It is now recognized that serum albumin is not a homogeneous material but can be fractionated by salting-out procedures (*cf.* pp. 163–164) into a number of distinct components all of which have albumin characteristics. The term *serum albumins* is preferable to the more widely used *serum albumin.* These proteins are, however, either not separated or but poorly separated by the widely used ultracentrifuge or electrophoretic methods. (See p. 461.)

Globulins are insoluble in salt-free water but soluble in neutral solutions of salts of strong bases with strong acids, such as NaCl, and are coagulable by heat; e.g., serum globulin, lactoglobulin, thyroglobulin, edestin from hemp seed, amandin from almond, and other vegetable globulins.

Serum globulin has been fractionated into several components by ultracentrifuge and electrophoretic methods (see Chapter 22). The three groups most easily identified are called α, β, and γ globulins. The γ-globulin fraction of human plasma is the source of almost all the immunologically active proteins of the blood. In fact, it appears that 50 per cent or more of the γ-globulin fraction prepared from pooled human plasma is composed of immunologically active material. During convalescence from illness, there is an increase in immune proteins in the plasma. This increase is accompanied by and accounted for by the increase in γ-globulin. Figure 57 shows a paper electrophoretic pattern of normal human serum.

Glutelins are simple proteins insoluble in all neutral solvents, but readily soluble in very dilute acids and alkalies; e.g., glutenin from wheat. These may be mixtures of denatured proteins.

Prolamins are simple proteins soluble in 70 to 80 per cent alcohol and insoluble in water, absolute alcohol, and other neutral solvents; e.g., zein from corn, gliadin from wheat and rye, hordein from barley, and bynin from malt. Rice, kafir, and sorghum have also been shown to contain alcohol-soluble proteins. The name prolamins was suggested for these alcohol-soluble vegetable proteins by Osborne, since upon hydrolysis they yield large amounts of proline and ammonia.

Albuminoids (**Scleroproteins**) comprise all fibrous proteins which have a supporting or protective function in the animal organism. In the plant kingdom the albuminoids are probably replaced by cellulose and similar substances.

1. COLLAGENS, the principal supporting proteins of skin, tendons, and bones, are resistant to peptic and tryptic digestion. They are converted into the easily digested soluble proteins, the gelatins, by boiling with water, dilute acids, or alkalies and contain large amounts of hydroxyproline.

2. ELASTINS, present in elastic tissues (tendons, arteries), are more readily digested by trypsin than by pepsin, are not convertable into gelatin, and give a negative or very weak test for hydroxyproline. Elastic tissue is a mixture of elastin, collagen, and a carbohydrate-containing protein, *elastomucin.* It is probable that many of the earlier studies

on "elastin" were really investigations on a mixture of elastin and elastomucin.[2]

3. KERATINS are proteins resistant to digestion by pepsin and trypsin, and insoluble in dilute acids and alkalies, in water, and in organic solvents. Keratins have been divided into two classes: (a) *Eukeratins* (true or hard keratins) are keratins which on hydrolysis yield histidine, lysine, and arginine in a molecular ratio of approximately 1:4:12,[3] and (b) *pseudokeratins* (false or soft keratins) are a heterogeneous group of insoluble proteins which are digestible with difficulty, and which do *not* yield histidine, lysine, and arginine in the molecular ratio of 1:4:12.

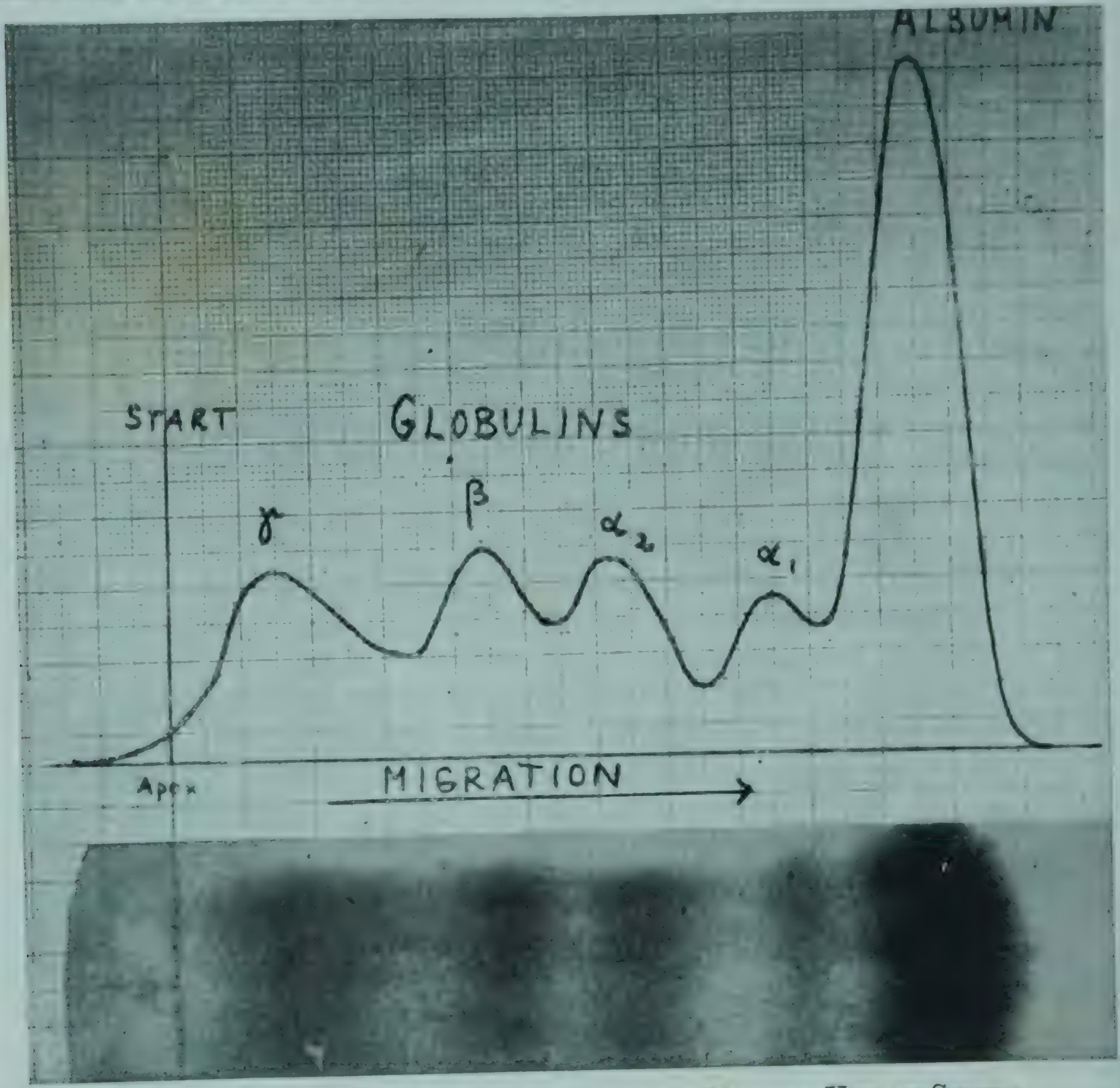

FIG. 57. PAPER STRIP ELECTROPHORESIS OF NORMAL HUMAN SERUM.

Lower portion, photograph of stained paper strip after electrophoresis; *upper portion*, curve obtained by photoelectric densitometry of the paper strip diagram.

Courtesy, K. G. Stern and C. V. Tondo, unpublished data.

Histones are soluble in water and insoluble in dilute ammonia, and, in the absence of ammonium salts, insoluble even in excess of ammonia. They yield precipitates with solutions of other proteins and a coagulum on heating which is easily soluble in very dilute acids. On hydrolysis they yield a large number of amino acids, among which arginine predominates. Histones contain tyrosine but appear to be lacking in tryptophan. In short, histones are basic proteins which stand between protamines and true proteins; e.g., scombrone from mackerel sperm, thymus histone.

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[2] Hall, Reed, and Tunbridge: *Nature*, **170**, 264 (1952).

[3] Since the development of better methods for the analysis of amino acids, these numerical values have changed but the principle still is valid.

Globins are simple basic proteins which were at one time classed as histones because of similarities in solubility, etc. The globins differ from the histones, however, in isoelectric point, toxicity, and amino acid composition. Histones are high in arginine and isoleucine and contain only traces of tryptophan, but globins contain average quantities of arginine and tryptophan and are unique in their high content of histidine and deficiency in isoleucine. Globins are usually found in nature as the protein portion of conjugated proteins, e.g., globin from hemoglobin.

Protamines are simpler polypeptides than the proteins included in the preceding groups. Their acid salts are soluble in water, uncoagulable by heat, have the property of precipitating aqueous solutions of other proteins, possess strong basic properties, and form stable salts with strong mineral acids. They yield comparatively few amino acids, among which the basic ones, especially arginine, predominate. They are the simplest natural proteins; e.g., salmine from salmon sperm, sturine from sturgeon sperm, clupeine from herring sperm, scombrine from mackerel sperm.

II. CONJUGATED PROTEINS

The conjugated proteins, like the simple proteins, are substances which may be isolated from biological materials by suitable methods. They differ from the simple proteins, however, in the fact that the intact molecule consists of protein combined with some nonprotein substance (the prosthetic group) in a manner which confers new and characteristic properties on the complex formed. They are classified according to the nature of the prosthetic group, as indicated below.

Nucleoproteins. Compounds of one or more protein molecules with nucleic acids. The nucleic acid of the cell nucleus appears to be deoxyribose nucleic acid which is united to the protamines, histones, and other basic proteins of the cell nucleus. The nucleoproteins of the cytoplasm yield ribose nucleic acid; the nature of the protein component awaits investigation.

Glycoproteins. Mucins contain a uronic acid (see Chapter 2) probably united in salt linkage to the basic groups of proteins. They are found in vitreous humor and in Wharton's jelly of the umbilical cord.

Mucoids do not contain a uronic acid but consist of protein firmly bound to a polysaccharide such as polymerized glucosamine-mannose; e.g., serum mucoid, ovomucoid.

Sulfomucins contain sulfuric acid, uronic acid, and either chondrosamine or glucosamine. They are found in cartilage, intestinal tissue, cornea, gastric mucosa.

Phosphoproteins. Compounds of the protein molecule with phosphoric acid other than a nucleic acid or lecithin;[4] e.g., casein from milk, ovovitellin from egg yolk, and other proteins associated with the feeding of the young. In the cell nucleus there are phosphoproteins the composition of which is still under investigation.

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[4] The accumulated chemical evidence distinctly points to the propriety of classifying the phosphoproteins as conjugated compounds, i.e.. they are esters of phosphoric acid (or acids) and protein.

Chromoproteins are compounds of proteins with a metal-containing prosthetic group, e.g., the red iron-containing hemoglobins (Fe = 0.34 per cent) from vertebrate blood; the blue copper-containing hemocyanins from the blood of certain invertebrates; the green magnesium-containing chlorophyll proteins from plants, etc. The protein should not be regarded as a mere colloidal carrier of the prosthetic group, but as an integral portion of the chromoprotein molecule, determining not only the magnitude but even the nature of the reaction it promotes. Thus one iron-containing prosthetic group combined with four different proteins gives rise to four different substances: methemoglobin, catalase, peroxidase, and a cytochrome (Fe = 0.43 per cent).

Lipoproteins are compounds of the protein molecule with lecithins, cholesterol, etc.

Metalloproteins are various protein enzymes which contain metals as an inherent portion of their molecule. Thus, tyrosinase contains copper, arginase contains manganese or magnesium, whereas zinc is present in carbonic anhydrase, and molybdenum in xanthine oxidase.

III. DERIVED PROTEINS

PRIMARY PROTEIN DERIVATIVES

The primary protein derivatives are substances formed from some of the simple and conjugated proteins on denaturation. When these proteins are subjected to certain chemical and physical agents they undergo intramolecular changes which are accompanied by changes in the properties of the original material. Although the nature of these intramolecular changes is still unknown, they may be recognized by the characteristic properties of the substances formed, as indicated in the classification below.

Proteans are insoluble products which apparently result from the incipient action of water, very dilute acids, or enzymes—e.g., fibrin from fibrinogen, myosan from myosin, edestan from edestin. This is probably an early denaturation stage.

Metaproteins are products of the action of dilute acids and alkalies whereby the molecule is so far altered as to form products soluble in weak acids and alkalies but insoluble at their isoelectric points; e.g., acid metaprotein (acid albuminate), alkali metaprotein (alkali albuminate).

Coagulated proteins are insoluble products which result when isoelectric solutions of the protein are denatured by (1) heat, (2) alcohol, (3) ultraviolet light, (4) ultrasonic waves, (5) mechanical shaking, (6) mineral acids and alkalies, (7) grinding, (8) neutral chemical agents, etc.

SECONDARY PROTEIN DERIVATIVES[5]

The secondary protein derivatives are substances formed during the hydrolysis of the protein molecule. As hydrolysis proceeds the intact molecule is split up into a series of smaller and smaller fragments which are designated, respectively, as primary and secondary proteoses, peptones, and peptides. The substances in each group are not homogeneous chemical entities, but rather mixtures of fragments of the original protein which probably vary in both composition and size and which are grouped together merely because they exhibit certain characteristic properties in common. This classification has little modern significance and it is being gradually abandoned except for the peptides.

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[5] The term secondary protein derivatives is used because their formation may be preceded by that of some of the primary derivatives.

Primary proteoses are soluble in water, noncoagulable by heat, precipitated by concentrated nitric acid, and precipitated by half-saturation with ammonium sulfate.

Secondary proteoses are soluble in water, noncoagulable by heat, and precipitated by saturating their solutions with ammonium sulfate.

Peptones are soluble in water, noncoagulable by heat, and not precipitated by saturating their solutions with ammonium sulfate.

Peptides are definitely characterized combinations of two or more amino acids, the carboxyl group of one being united with the amino group of the other with the elimination of a molecule of water,[6]—e.g., dipeptides, tripeptides, tetrapeptides, pentapeptides, etc.

CONSIDERATION OF VARIOUS CLASSES OF PROTEINS

I. SIMPLE PROTEINS

A. ALBUMINS

Albumins constitute the first class of simple proteins and may be defined as simple proteins which are coagulable by heat and soluble in pure (salt-free) water. They are also soluble in salt solutions and those of animal origin are not precipitated upon saturating their solutions at 30°C. with sodium chloride or magnesium sulfate except in the vicinity of their isoelectric points. All albumins of animal origin are soluble in half-saturated ammonium sulfate solution[7] but may be precipitated by increasing the salt concentration up to full saturation. They may also be thrown out of solution by the addition of a sufficient quantity of a mineral acid. Metallic salts also possess the property of precipitating albumins, some of the precipitates being soluble in excess of the reagent, whereas others are insoluble in such an excess. Many albumins have been obtained in crystalline form, notably egg and serum albumins from various species.

EXPERIMENTS ON ALBUMINS

Besides the general protein color reactions and precipitation reactions described in the previous chapter, the albumins have other properties which are used to identify proteins belonging to this class. Some of these properties are illustrated by the following experiments:

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[6] The peptones are undoubtedly peptides or mixtures of peptides, the latter term being at present used to designate those of definite structure.

[7] In this connection Osborne's observation is of interest, namely that certain vegetable albumins are precipitated by saturating their solutions with sodium chloride or magnesium sulfate or by half-saturating with ammonium sulfate.

1. *Solubility in Concentrated Salt Solutions.* (a) Place 25 ml. of dilute egg albumin solution (prepared as described on p. 169) in a beaker and add 6.6 g. of solid ammonium sulfate. Stir until dissolved. The solution is now 2 M in ammonium sulfate, or approximately half-saturated. Is the albumin precipitated? Now add an excess of solid ammonium sulfate and stir to produce full saturation with the salt. What happens? Dilute a small portion of the mixture with distilled water. Is the effect of ammonium sulfate reversible? Filter the remainder of the mixture. Test the precipitate by Millon's test. Test the filtrate for protein by the biuret test, using saturated ammonium sulfate solution as a control. What are your conclusions?

(b) To a 25-ml. portion of egg albumin solution in a beaker add an excess of solid sodium chloride and stir until the solution is saturated with the salt. How does the result differ from that with ammonium sulfate? Add 2 to 3 drops of acetic acid. What occurs? Explain.

2. *Heat Coagulation.* Add 3 to 5 drops of acetic acid to 25 ml. of dilute egg albumin solution in a small evaporating dish and heat to boiling. What happens? Why is the acetic acid added? Test a portion of the coagulum by Millon's reaction. Explain.

B. GLOBULINS

Globulins are simple proteins present in blood serum, muscle, and other animal tissues, and also present in many plant seeds. They give all the ordinary protein tests and are coagulable by heat. Globulins differ from the albumins in being insoluble in pure (salt-free) water. They are, however, soluble in neutral solutions of the *salts* of strong acids with strong bases, such as sodium chloride. The globulins require a certain concentration of salt in order that they may remain in solution, precipitating when the concentration of salt is lowered by such processes as dilution or dialysis. In general the globulins are precipitated by half-saturation of their solutions with ammonium sulfate—i.e., by the addition to their solutions of an equal volume of saturated ammonium sulfate solution.[8] Most globulins are also precipitated from their solutions by saturation with solid sodium chloride or magnesium sulfate.

Blood serum apparently contains a variety of globulins, characterized by differences in solubility, in precipitability by ammonium or sodium sulfate, and in rate of electrophoretic migration (Chapter 22). Blood globulins have not yet been crystallized but crystalline globulins have been obtained from milk (β-lactoglobulin), muscle (myosin), gastric juice (pepsin), and numerous plant seeds.

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[8] It is generally stated that globulins are precipitated from their solutions upon half-saturation with ammonium sulfate and that albumins are precipitated upon complete saturation by this salt. Comparatively few exceptions were found to this rule until proteins of vegetable origin came to be more extensively studied. These studies, furthered especially by Osborne and associates, have demonstrated very clearly that the characterization of a globulin as a protein which is precipitated by half-saturation with ammonium sulfate can no longer hold. Certain vegetable globulins have been isolated which are not precipitated by this salt until a concentration is reached greater than that secured by half-saturation. As an example of an albumin which does not conform to the definition of an albumin as regards its precipitation by ammonium sulfate may be mentioned the leucosin of the wheat germ, which is precipitated from its solution upon half-saturation with ammonium sulfate. The limits of precipitation by ammonium sulfate, therefore, do not furnish a sufficiently accurate basis for the differentiation of globulins from albumins.

We have used an albumin of animal origin (egg albumin) for all the protein tests thus far, whereas the globulin to be studied will be prepared from a vegetable source. The vegetable globulin we shall study may be taken as a type of all globulins, both animal and vegetable.

EXPERIMENTS ON GLOBULIN

Preparation of Edestin. **Extract 20 to 30 g. (a handful) of crushed hemp seed with about 150 ml. of 10 per cent sodium chloride solution for one-half hour at 60° C. Filter while hot through a filter paper moistened with 10 per cent sodium chloride solution into a 600-ml. beaker. To the warm filtrate, carefully add distilled water heated to 60° C. until the solution just becomes turbid (300 to 500 ml. of water are required, depending upon the concentration of the protein). Warm the solution in a water bath at 60° until it becomes clear and then permit both the solution and the water bath to cool spontaneously at room temperature. In 24 hours there settles out a precipitate of globulin which is almost entirely crystalline in form. This particular globulin in hemp seed is called edestin. It is soluble in warm 10 per cent sodium chloride solution and may be crystallized by cooling its solution, or by dialyzing the solution against distilled water. Addition of warm water, as above, increases the yield of crystals obtained by decreasing the solubility of the protein. Edestin crystallizes in several different forms, chiefly octahedra (see Fig. 58). Filter off the edestin and make the following tests on the crystalline material.**

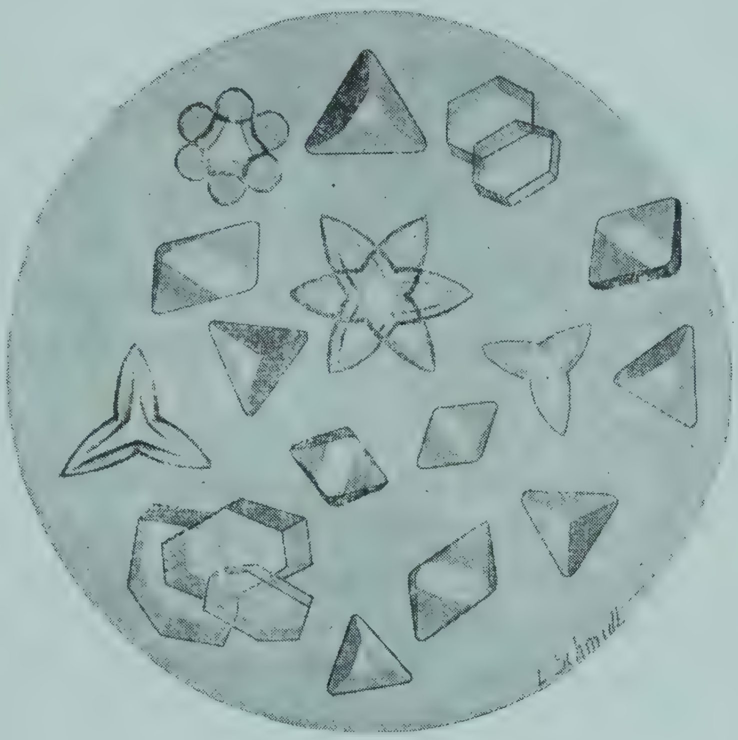

FIG. 58. EDESTIN.

Tests on Crystallized Edestin.

1. Microscopical Examination **(see Fig. 58).**

2. Solubility. **Try the solubility in water, 1 per cent sodium chloride solution, dilute acid and alkali, and alcohol.**

3. Millon's Reaction.

4. *Coagulation Test*. Place a small amount of the globulin in a test tube, add a little water, and boil. Now add hydrochloric acid and note that the protein no longer dissolves. It has been coagulated.

Dissolve the remainder of the edestin in about 50 ml. of 10 per cent sodium chloride solution, and make the following tests on this solution.

Tests on Edestin Solution.

1. *Influence of Protein Precipitants.* Try a few protein precipitants such as nitric acid, tannic acid, picric acid, and mercuric chloride. Compare with the results on egg albumin (p. 174). Can you distinguish between albumin and globulin by tests such as these?

2. *Biuret Test.*

3. *Coagulation Test.* Boil some of the solution in a test tube. What happens?

4. *Saturation with Sodium Chloride.* Saturate about 10 ml. of the solution with solid sodium chloride. How does this result differ from that obtained upon saturating egg albumin solution with solid sodium chloride?

5. *Precipitation by Dilution.* Add a few drops of the solution to a test tube filled with distilled water. Why does the globulin precipitate?

6. *Precipitation by Dialysis.* Place the remainder of the solution in a dialyzing bag and dialyze against about 500 ml. of distilled water. Why does the globulin precipitate? Examine the precipitate under the microscope.

C. GLUTELINS

It has been repeatedly shown, particularly by Osborne, that after extracting the seeds of cereals with water, neutral salt solution, and strong alcohol, there still remains a residue which contains protein material which may be extracted by very dilute acid or alkali. These probably denatured proteins which are insoluble in all neutral solvents, but readily soluble in very dilute acids and alkalies, are called glutelins. The only member of the group which has yet received a name is the glutenin of wheat,[9] a mixture of proteins which constitutes nearly 50 per cent of the gluten, the remainder being principally gliadin.

Gluten: Preparation and Tests. To about 50 g. of wheat flour in a casserole or evaporating dish add a little water and mix thoroughly until a stiff dough results. Knead this dough thoroughly and permit it to stand for about half an hour. This is done in order that the maximum quantity of gluten may be obtained. Treat the dough with about 200 ml. of water and knead it thoroughly. Note the yellowish color of the dough and the milky appearance of the water due to suspended starch granules. (Place a drop of the suspension on a slide, cover with a cover slip, run underneath the slip a drop of iodine solution and observe the stained starch granules under the microscope.)

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[9] Work by Blish and Sandstedt indicates that glutenin prepared by extraction of gluten with dilute alkali is actually an irreversible alteration product of a more complex protein in the crude gluten.

Filter and apply a protein color reaction (see p. 169) to the filtrate. It should be positive, indicating that water-soluble proteins were present in the flour. Add fresh water to the dough and repeat the kneading process. Continue this procedure with fresh addition of water, or holding the gluten under the tap, until practically no starch granules are noted in suspension. To a small piece of the yellow, fibrous gluten apply Millon's reaction (p. 169). This test shows gluten to be protein material. Utilize the remainder of the gluten in the preparation of gliadin below.

Glutenin: Preparation and Tests. **(In the preparation of gliadin below it is customary to remove this prolamin from the crude gluten by extracting with 70 per cent alcohol. Inasmuch as gluten consists chiefly of gliadin and glutenin the portion of the gluten remaining after the extraction of the alcohol-soluble protein gliadin may be utilized for the preparation of glutenin.)**

To the finely divided residue from the preparation of gliadin below in a flask or bottle add about 250 ml. of 70 per cent alcohol. Allow to stand for about 48 hours with repeated shaking in order to remove any remaining gliadin. Crude glutenin remains. To purify the glutenin, treat it in a mortar with sufficient 0.2 per cent NaOH to dissolve it, and filter the liquid through a wet pleated filter. Neutralize the filtrate carefully with 0.2 per cent HCl, adding the acid drop by drop with thorough mixing after each addition. (The glutenin is soluble in excess of acid.) Filter off the glutenin precipitate and wash several times with 70 per cent alcohol and finally with water. Apply the following tests:

1. Solubility in water, 0.9 per cent NaCl solution, 0.2 per cent HCl, and 0.5 per cent Na_2CO_3.

2. Millon's Reaction.

D. PROLAMINS (ALCOHOL-SOLUBLE PROTEINS)

The term prolamin was proposed by Osborne for the group of plant proteins formerly termed "alcohol-soluble proteins." The name is very appropriate inasmuch as these proteins yield, upon hydrolysis, especially large amounts of proline and ammonia. The prolamins are simple proteins which are insoluble in water, absolute alcohol, and other neutral solvents, but are soluble in 70 to 80 per cent alcohol and in dilute acids and alkalies. They occur widely distributed, particularly in the vegetable kingdom, and include zein of maize, hordein of barley, gliadin of wheat and rye, bynin of malt, and kafirin of kafir. Sorghum and rice also contain alcohol-soluble proteins. They yield relatively large amounts of glutamic acid on hydrolysis but little or no lysine. The largest percentage of glutamic acid (47 per cent) ever obtained as a decomposition product of a protein substance was obtained from the hydrolysis of the prolamin gliadin. This yield of glutamic acid is also the largest amount of any single decomposition product yet obtained from any protein except the 88.4 per cent of arginine obtained from salmin.

Gliadin: Preparation and Tests. **Introduce the finely divided crude gluten as prepared on p. 190 into a flask or bottle, add about 250 ml. of 50 to 70 per cent alcohol, and allow the mixture to stand 24 hours with occasional shaking. Filter (retaining the undissolved portion for preparation of glutenin, above) and evaporate the filtrate to dryness in a porcelain dish over a water**

bath. Pulverize the dry material. Apply the following tests to this gliadin powder:

Solubility and Protein Tests. **Test the solubility in alcohol (30 per cent, 50 per cent, and 70 per cent), water, 0.9 per cent NaCl, 0.2 per cent HCl, and 0.5 per cent Na_2CO_3. Shake each tube repeatedly and filter. To the filtrate apply the coagulation test (p. 190) and the biuret test (p. 171).**

E. Albuminoids (Scleroproteins)

The albuminoids yield hydrolytic products similar to those obtained from the other simple proteins already considered, thus indicating that they possess essentially the same chemical structure. They differ from all other proteins, whether simple, conjugated, or derived, in that they are insoluble in all neutral solvents. The albuminoids include "the principal organic constituents of the skeletal structure of animals as well as their external covering and its appendages." Some of the principal albuminoids are keratins from hair, horn, nails, feathers, etc.; elastins from arteries, ligaments; collagens from bone, skin, hoof, etc.; reticulin, spongin, and silk fibroin. Gelatin cannot be classed as an albuminoid although it is a transformation product of collagen. The various albuminoids differ from each other in certain fundamental characteristics (see p. 183). Experiments on albuminoids will be found in Chapter 9.

II. CONJUGATED PROTEINS

Conjugated proteins consist of a protein molecule united to some nonprotein molecule or molecules otherwise than as a salt. Glycoproteins, nucleoproteins, chromoproteins, phosphoproteins, lipoproteins, and metalloproteins are the six classes of conjugated proteins.

Glycoproteins may be considered as compounds of the protein molecule with a substance or substances containing a carbohydrate group other than a nucleic acid. They yield, upon decomposition, protein and carbohydrate derivatives, notably glucosamine, $CH_2OH\cdot(CHOH)_3\text{-}CH(NH_2)\cdot CHO$, galactosamine, galactose, fucose, and uronic acids. The principal glycoproteins are (1) mucoids, (2) mucins, and (3) sulfomucins. The elementary composition of typical mucoids is as follows:

	N	*S*	*C*	*H*	*O*
Tendomucoid	11.75	2.33	48.76	6.53	30.60
Osseomucoid	12.22	2.32	47.43	6.63	31.40

(For the preparation of tendomucoid see Chapter 9; of salivary mucin, see Chapter 13.) Amyloid,[10] which appears pathologically in the spleen, liver, and kidneys, is also a glycoprotein.

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[10] Not to be confused with the substance amyloid, which may be formed from cellulose (see p. 90).

Phosphoproteins are considered to be compounds of the protein molecule and phosphoric acid. The percentage of phosphorus in phosphoproteins is very similar to that in nucleoproteins, but they differ from this latter class of proteins in that they do not yield any purine bases upon hydrolytic cleavage. Two of the common phosphoproteins are the casein of milk and the ovovitellin of egg yolk. The phosphorus in these proteins exists in phosphoric acid radicals bound in ester linkage to the hydroxy amino acids serine and threonine. For the preparation of a typical phosphoprotein (casein), see Chapter 8.

Chromoproteins are compounds of the protein molecule with a metalloporphyrin (see Chapter 22) or some similar substance. The principal members of the group are the hemoglobin of the blood and the chlorophyll proteins of plants. Upon hydrolytic cleavage, hemoglobin yields a protein termed globin and a coloring matter which contains iron and is known as heme. Hemocyanin, another member of the class of chromoproteins, occurs in the blood of certain invertebrates, notably cephalopods, gasteropods, and crustacea. Hemocyanin generally contains either copper, manganese, or zinc, as contrasted to the iron of the hemoglobin molecule. For the preparation of hemoglobin in crystalline form, see Chapter 22.

Lipoproteins consist of a protein molecule joined to lecithin, cholesterol, and other fatty substances. They have been comparatively little studied and may possibly be mixtures of protein and lipide material. These unions are not split by treatment with ether but are by alcohol, especially after heat denaturation of the protein.

Nucleoproteins. For consideration of nucleoproteins, see Chapter 7.

Metalloproteins. Numerous protein enzymes contain metals as an inherent portion of their molecules; e.g., carbonic anhydrase is a zinc protein complex, arginase contains manganese or magnesium, tyrosinase contains copper, etc.

III. DERIVED PROTEINS

A. PRIMARY PROTEIN DERIVATIVES

1. PROTEANS

Proteans are those insoluble, denatured protein substances which are produced from proteins originally soluble through the incipient action of water, enzymes, very dilute acids, etc. According to Osborne nearly all proteins may give rise to proteans, and the determining factor in the transformation is the hydrogen-ion concentration. The protean produced from the transformation of edestin is called edestan; that produced from myosin is called myosan, etc. The name protean was first given to this class of proteins by Osborne in 1900 in connection with his studies of edestin. It is but little used at present.

2. METAPROTEINS

The metaproteins are denatured proteins formed by the action of dilute acids and alkalies on some proteins, especially on the albumins and the globulins. The conversion of protein into metaprotein is accelerated by a rise in temperature, taking place almost immediately at the boiling point. There is, at present, no definite evidence concerning the exact nature of the changes which the protein molecule undergoes during this transformation. (See the section "Protein Denaturation and Its Reversal" in Chapter 5.) The denatured protein behaves like a suspensoid, dissolving in dilute acids and alkalies—i.e., when the particles are electrically charged—and flocculating when the solutions are brought to the isoelectric point. The suspensoid character of the metaproteins is also indicated by their sensitivity to electrolytes, especially in the neighborhood of the isoelectric point. The metaproteins are precipitated by saturation of their solutions with ammonium sulfate and, when dissolved in acid, by full saturation with sodium chloride. Boiling an isoelectric suspension of metaprotein converts it into coagulated protein, in which case the material is no longer soluble in dilute acids and alkalies.

The metaproteins are usually classified as (1) acid metaproteins, or so-called acid albuminates, and (2) alkali metaproteins, or alkali albuminates, depending upon whether they are formed by the action of acid or alkali on the native protein. Transformation products with similar physical properties are formed by the action of enzymes, ultraviolet light, etc., on native protein. It is unlikely that these procedures ever produce a homogeneous metaprotein.

EXPERIMENTS ON A DENATURED PROTEIN

Preparation and Study: Alkali Metaprotein (Alkali Albuminate). **Carefully separate the white from the yolk of a hen's egg and place the former in an evaporating dish. Add 10 per cent sodium hydroxide solution, drop by drop, stirring continuously. The mass gradually thickens and finally assumes the consistency of jelly. This is solid alkali metaprotein or "Lieberkühn's jelly." Do not add an excess of sodium hydroxide or the jelly will dissolve. Cut it into small pieces, place a cloth or wire gauze over the dish, and by means of running water, wash the pieces free from adherent alkali. Now add a small amount of water, which forms a weak alkaline solution with the alkali within the pieces, and dissolve the jelly by gentle heat. Cool the solution and divide it into two parts. Proceed as follows with the first part: Neutralize with dilute hydrochloric acid, noting the odor of the liberated hydrogen sulfide as the alkali metaprotein precipitates. Filter off the precipitate and test as follows:**

1. *Solubility.* Solubility in water, sodium chloride solution, dilute acid, and alkali.

2. *Millon's Reaction.*

3. *Coagulation Test.* Suspend a little of the metaprotein in water (neutral solution) and heat to boiling for a few moments. Now add 1 to 2 drops of dilute NaOH solution to the water and see if the metaprotein is still soluble in dilute alkali. What is the result and why?

4. *Test for Cystine and Cysteine Sulfur* **(see p. 168).**

Subject the second part of the original solution to the following tests:

5. *Coagulation Test.* Heat some of the solution to boiling in a test tube. Does it coagulate?

6. *Biuret Test.*

7. *Influence of Protein Precipitants.* Try a few protein precipitants such as picric acid and mercuric chloride. How do the results obtained compare with those from the experiments on egg albumin? (See p. 174.)

3. COAGULATED PROTEINS

Simple proteins, such as the albumins and globulins, are converted by heat, ultraviolet light, mechanical agitation, or long contact with alcohol, etc., into insoluble materials known as coagulated proteins, which probably consist of the matting together of the denatured protein fibrils similar to the formation of felt from short fibers of hair. (See p. 156 and the section "Protein Denaturation and Its Reversal" in Chapter 5.) Coagulated proteins are insoluble in water, salt solutions, and dilute acids and alkalies. They are soluble in strong mineral acids and alkalies which hydrolyze the protein into simpler substances.

EXPERIMENTS ON COAGULATED PROTEIN

Ordinary coagulated egg white may be used in the following tests:

1. *Solubility.* Try the solubility of small pieces of coagulated protein in water, 1 per cent sodium chloride, and dilute and concentrated acid and alkali.

2. *Millon's Reaction.*

3. *Xanthoproteic Reaction.* Partly dissolve a medium-sized piece of the protein in concentrated nitric acid. Cool the solution and carefully add an excess of ammonium hydroxide. Both the protein solution and the undissolved protein will be colored orange.

4. *Biuret Test.* Partly dissolve a medium-sized piece of the protein in concentrated sodium hydroxide solution. If the proper dilution of copper sulfate solution is now added, the white coagulated protein, as well as the protein solution, will assume the characteristic purplish-violet color.

B. SECONDARY PROTEIN DERIVATIVES

1. PROTEOSES AND PEPTONES

The proteoses and peptones are poorly defined intermediate products formed during the digestion of proteins by the proteolytic enzymes, as well as in the decomposition of proteins by hydrolysis and the putrefaction of proteins through the action of bacteria. As hydrolysis proceeds, the large, colloidal protein molecule is split up into a mixture of large and small fragments which were formerly designated as primary and secondary proteoses, peptones, and peptides. The larger fragments are constantly being broken down until, finally, only amino acids remain. It should be emphasized that the substances formerly known as proteoses and peptones are not homogeneous chemical entities, but rather mixtures of fragments of the original protein molecule which vary in composition and also in size, and are grouped together merely because they exhibit certain characteristic properties in common. Those fragments which are precipitated when the solution is half-saturated with ammonium sulfate were known as primary proteoses; those precipitated when the solution is saturated with ammonium sulfate were called secondary proteoses. The peptones were not precipitated by ammonium sulfate. The proteoses still exhibit colloidal properties, but these properties become less pronounced and begin to disappear entirely by the time the breakdown of the protein molecule has reached the peptone stage. Thus, in addition to being nonprecipitable by ammonium sulfate, the peptones diffused through such membranes as collodion and failed to give some of the characteristic precipitation reactions of proteins.

Since the proteoses and peptones are both heterogeneous mixtures of fragments of the original protein molecules, different preparations of these substances will show variations in composition and properties depending upon the nature of the protein used as starting material, the method of hydrolysis employed, and the method used to separate the various products of hydrolysis. Thus the so-called peptones sold commercially vary not only in composition but also in the amounts of primary and secondary proteoses they contain. As a class the proteoses and peptones are a mixture of amino acids and peptides not coagulated by heat. Some are also soluble in dilute alcohol. *Peptones differ from proteoses in being more diffusible, and nonprecipitable by* $(NH_4)_2SO_4$, *and by their failure to give any reaction with potassium ferrocyanide and acetic acid, potassio-mercuric iodide and HCl, picric acid, and trichloroacetic acid.* Peptones may be precipitated by phosphotungstic acid, phosphomolybdic acid, absolute alcohol, and tannic acid, but an excess of the precipitant may dissolve the precipitate. The so-called primary proteoses, being relatively large molecules, are precipitated by HNO_3 and are the only members of the proteose-peptone group which are so precipitated.

EXPERIMENTS ON PROTEOSES AND PEPTONES

Some of the more general characteristics of the proteose-peptone group may be noted by making the following simple tests on a proteose-peptone powder:

1. *Solubility.* Solubility in hot and cold water and sodium chloride solution.

2. *Millon's Reaction.*

3. *Precipitation by Picric Acid.* To 5 ml. of proteose-peptone solution in a test tube add picric acid until a permanent precipitate forms. The precipitate disappears on heating and returns on cooling.

4. *Precipitation by a Mineral Acid.* Try the precipitation by nitric acid.

5. *Coagulation Test.* Heat a little proteose-peptone solution to boiling. Does it coagulate like the other simple proteins studied?

SEPARATION OF PROTEOSES AND PEPTONES[11]

Place 50 ml. of proteose-peptone solution in an evaporating dish or casserole, and half-saturate it with ammonium sulfate solution, which may be accomplished by adding an equal volume of saturated ammonium sulfate solution. At this point note the appearance of a precipitate of the primary proteoses. Now heat the half-saturated solution and its suspended precipitate to boiling and saturate the solution with solid ammonium sulfate. At full saturation the secondary proteoses are precipitated. The peptones remain in solution.

Proceed as follows with the precipitate of proteoses: Collect the sticky precipitate on a rubber-tipped stirring rod or remove it by means of a watch glass to a small evaporating dish and dissolve it in a little water. To remove the ammonium sulfate, which adhered to the precipitate and is now in solution, add barium carbonate, boil, and filter off the precipitate of barium sulfate. Concentrate the proteose solution to a small volume[12] and make the following tests:

Tests on Proteoses.

1. *Biuret Test.*

2. *Precipitation by Nitric Acid.* What would a precipitate at this point indicate?

3. *Precipitation by Trichloroacetic Acid.* This precipitate dissolves on heating and returns on cooling.

4. *Precipitation by Picric Acid.* This precipitate also disappears on heating and returns on cooling.

5. *Precipitation by Potassio-mercuric Iodide and Hydrochloric Acid.*

6. *Coagulation Test.* Boil a little in a test tube. Does it coagulate?

7. *Precipitation by Acetic Acid and Potassium Ferrocyanide.*

Tests on Peptones. The solution containing the peptones should be cooled and filtered, and the ammonium sulfate in solution removed by boiling with barium carbonate as described above. After filtering off the barium sulfate precipitate in the presence of a filter aid such as diatomaceous earth, concentrate the peptone filtrate to a small volume and repeat the tests as given under the proteose solution, above. Also try the precipitation by tannic acid. In the biuret test the solution should be made very strongly alkaline with solid potassium hydroxide.

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[11] The separation of proteoses and peptones by means of fractional precipitation with ammonium sulfate does not possess the significance it was once supposed to possess inasmuch as the boundary between these substances and peptides is not well defined (see p. 113). For discussion of a quantitative method for determining protein and proteose based on their separation by trichloroacetic acid, see Seibert: *J. Biol. Chem.*, **70**, 265 (1926).

[12] If the proteoses are desired in powder form, this concentrated proteose solution may now be precipitated by alcohol or acetone, and this precipitate, after being washed with acetone and with ether, may be dried and powdered.

SCHEME FOR DETECTION OF PROTEINS

If the solution is acid or alkaline it should be *approximately* neutralized. If a precipitate forms it should be filtered off. The neutralization need not necessarily proceed until an exact neutral reaction is obtained but should cease at the point where the largest precipitate is secured.

- Precipitate indicates
    - *Acid metaprotein* or *protean*, if the unknown solution was acid in reaction. (Test the precipitate by protein color tests.) It is practically impossible in the light of our present knowledge to differentiate between the *proteans* and the *metaproteins*.
    - *Alkali metaprotein, mucin* (*mucoid*) or *phosphoprotein*, if the unknown solution was alkaline in reaction.
        - *Alkali metaprotein*, if the precipitate is *very easily* soluble in an excess of acetic acid. (Test the metaprotein by protein color tests.)
        - *Phosphoprotein* or *nucleoprotein*, if the precipitate is *fairly* soluble in an excess of acetic acid. (Test the precipitate by protein color tests.) Also test the precipitate for phosphorus by test on p. 212. Hydrolyze and test for *purine bases* according to method given in Chapter 7. Positive reaction indicates *nucleoprotein*.
        - *Mucin* or *mucoid*, if the precipitate is soluble with difficulty in an excess of acetic acid. Hydrolyze some of the precipitate and test by Benedict's solution. See p. 66.
- Filtrate. This may contain *albumin, globulin, proteose, peptone*, and *gelatin*.

    Neutralize the filtrate, heat to boiling, and acidify slightly. If there is any coagulation note the temperature at which it occurs. Filter off the coagulum.
    - Coagulum indicates *albumin* or *globulin* or both. (Test by protein color tests.)

        Remove the metaprotein from some of the unknown solution, if any is present, and *half saturate* the filtrate with $(NH_4)_2SO_4$. (By adding an equal volume of *saturated* $(NH_4)_2SO_4$ solution.) If a precipitate forms, filter it off.
        - Precipitate indicates *globulin, primary proteose*, or *gelatin*. (Try protein color tests.)

            Separate globulin by pouring some of the unknown solution into 20 to 40 volumes of water.
            - Precipitate indicates *globulin*.
            - No precipitate indicates *primary proteose* or *gelatin*.*
        - Filtrate. *Saturate* with *solid* $(NH_4)_2SO_4$. Precipitate indicates *secondary proteose* or *albumin*. Filter it off and dissolve in *warm water*.

            Heat to boiling
            - Coagulation indicates *albumin*.
            - No coagulation indicates *secondary proteose*. (Try protein color test.)
    - Filtrate. *Half saturate* with $(NH_4)_2SO_4$.
        - Precipitate indicates *primary proteose* or *gelatin*.
        - Filtrate. *Saturate hot* with $(NH_4)_2SO_4$.
            - Precipitate indicates *secondary* proteose.

                Dissolve the precipitate in warm water* and saturate this neutral solution with $MgSO_4$.
                - Precipitate indicates *gelatin*. (Try any gelatin tests given in Chapter 9.)
                - Filtrate may contain *secondary proteose*. (Try any tests given on p. 197.)
            - Filtrate. Cool and filter off the $(NH_4)_2SO_4$.

                Test for *peptone* by the biuret test. (Use an excess of *solid* KOH.)

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* Gelatin and proteose may also be differentiated by means of the Hopkins-Cole reaction (see p. 170). A positive reaction here would indicate proteose and a negative reaction gelatin.

Model Chart for Review Purposes

Protein	*Solubility*						*Protein Color Test*	*Precipitation Tests*							*Salting-out Tests*		*Diffusion*	*Coagulation by Heat*
	Water	*10% NaCl*	*0.2% HCl*	*0.5% Na_2CO_3*	*Conc. HCl*	*Conc. KOH*		*Mineral Acid (HNO_3)*	*Metallic Salt ($HgCl_2$)*	*Alcohol*	*Pot. Ferrocyanide + Acetic Acid*	*Potassio-mercuric Iodide + HCl*	*Picric Acid*	*Trichloroacetic Acid*	*$(NH_4)_2SO_4$*	*NaCl*		
Albumin																		
Globulin																		
Nucleoprotein																		
Phosphoprotein																		
Glycoprotein																		
Acid metaprotein																		
Alkali metaprotein																		
Proteose																		
Peptone																		
Coagulated protein																		

2. PEPTIDES

The peptides are "definitely characterized combinations of two or more amino acids, the carboxyl (COOH) group of one being united with the amino (NH_2) group of the other with the elimination of a molecule of water." These peptides are more fully discussed on pp. 113 and 152.

REVIEW OF PROTEINS

In order to facilitate the student's review of the proteins, the preparation of a chart similar to the model shown above is recommended. The signs + and − may be conveniently used to indicate positive and negative reactions.

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7

Nucleic Acids and Nucleoproteins

Nucleoproteins owe their name to the fact that the first representatives of this class of conjugated proteins were obtained from cell nuclei. In the 1870's the Swiss chemist, Miescher, influenced by the growing importance of the nucleus in the cell theory, began the investigation of its composition and recognized a new type of compound which he termed *Nucleinstoff*. This proved to be a conjugate of protein and an acidic material of high molecular weight now known as nucleic acid. It has developed that compounds of this general chemical type are far more widely distributed than the name implies.

Distribution. Two major types of nucleic acid are now recognized in all cells, whether plant, animal, or bacterial. These are generally differentiated by the kind of carbohydrate present, *2-deoxypentose* or *pentose*. In normal cells the *deoxypentose nucleic acid* is found only in the nucleus in association with the chromosomal material, and probably is the only substance truly deserving the name *nucleic acid* in the sense implied by Miescher. The bulk of the *pentose nucleic acids* are in the cytoplasm. Small amounts of pentose nucleic acids are also present in the nucleus, both in the nucleoli and in the chromosomal material as isolated by the procedures of Mirsky.

Cytochemical and biological developments of the last two or three decades have focused attention on the overwhelming circumstantial evidence which associates deoxypentose nucleic acid with the morphological forms recognized as the chromosomes. The facts that viruses and bacteriophages have invariably proved to contain one or both types of the nucleoproteins, and that the simplest ones are pure crystalline nucleoproteins, have pointed to some unique function of nucleoproteins in directing growth and reproductive processes. Of special significance are certain substances which have the property of bringing about an inheritable alteration (mutation) of living bacteria, such as that from pneumococcus type II rough to type III smooth. These agents, the *transforming factors*, exhibit the kind of ability which is usually associated only with genes. In some instances such substances have been shown to be essentially pure deoxypentose nucleic acid.

Characteristics of Nucleoproteins. In all cases the nucleic acids have been found to be associated with protein. In some of the pentose nucleoproteins the association is very firm and appears to be in the nature of a covalent linkage. The deoxypentose nucleic acids, however, are found as salts of the strongly basic proteins, the histones. In the case of sperm nuclei they are also found with basic protamines of low molecular weight, which may contain up to 85 per cent of arginine. An unusual characteristic of the deoxypentose nucleoproteins is that they are less soluble in physiological saline solution than in either strong salt solutions or pure water. The insoluble material is highly fibrous and can be collected by winding it around a stirring rod.

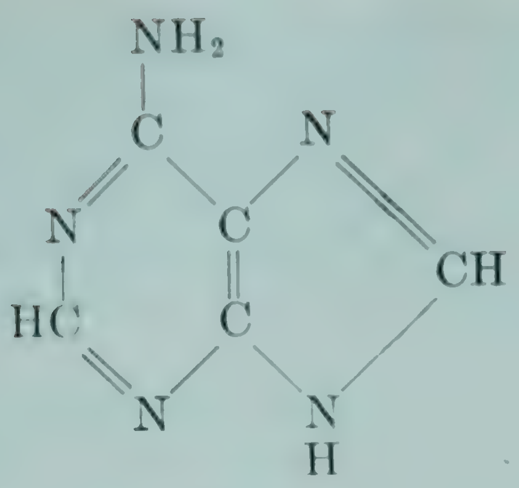
When separated from the protein the nucleic acids are sparingly soluble in cold water, insoluble in alcohol, but readily soluble in weak alkali with the formation of the alkali metal salts. They are precipitated from alkaline solutions by hydrochloric acid, but only the pentose nucleic acids are precipitated by acetic acid. In weakly acidic solution the nucleic acids can be precipitated by some proteins, the combination being effectively a nucleoprotein. They form insoluble salts with alkaline earth and heavy metals. When sufficiently purified they do not give the protein color reactions.

Molecular Size. Pentose nucleic acid preparations have been demonstrated, by ultracentrifugal measurement of their sedimentation rates, to have molecular weights from a few thousand up to 300,000; however, it is very difficult to avoid degradation in the process of isolation and few samples are available with the higher molecular weights. Similar measurements show molecular weights of deoxypentose nucleic acid to be up to 1,000,000 or 2,000,000, although there is some indication that the particles actually measured may be aggregates of materials of somewhat lower molecular weights. The sodium salt of the deoxypentose nucleic acid can yield a clear solution while warm, but this solidifies to a gelatinous mass upon cooling, which can be again liquefied by warming. When such gels are allowed to flow, or are stirred in a beaker, the gel partially liquefies, and under polarized light the existence of flow birefringence can be seen. This phenomenon is due to the presence of long, slim, fibrous molecules which line up in parallel in the moving solution, but again distribute themselves at random when motion of the solution ceases.

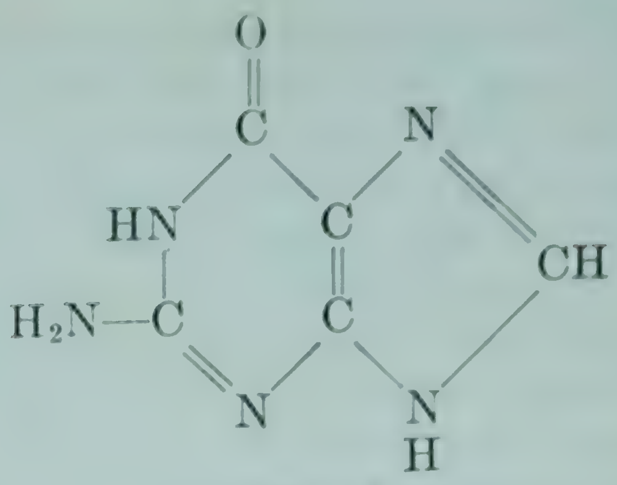
THE COMPOSITION OF THE NUCLEIC ACIDS

The nucleic acids are composed of nitrogenous heterocyclic bases (the purines and pyrimidines), carbohydrates, and phosphoric acid. The carbohydrates, where identified, have always proved to be the pentoses, D-ribose ($CHO{\cdot}CHOH{\cdot}CHOH{\cdot}CHOH{\cdot}CH_2OH$), or D-2-deoxyribose ($CHO{\cdot}CH_2{\cdot}CHOH{\cdot}CHOH{\cdot}CH_2OH$) and instead of the more general terms pentose and deoxypentose the names *ribose nucleic acid* and *deoxyribose nucleic acid*, frequently abbreviated to *RNA* and *DRNA* or *DNA*, are widely used.

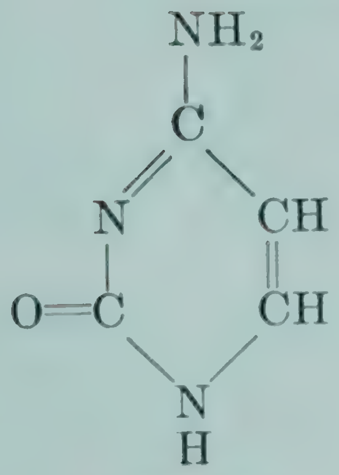
The Purines and Pyrimidines. The two purines adenine and guanine and the pyrimidine cytosine are found in both types of nucleic acid. The pyrimidine uracil has been found only in the ribose nucleic acids, and thymine (5-methyl-uracil) is characteristic of deoxyribose nucleic acids. Two other pyrimidines, 5-methyl-cytosine and 5-hydroxymethyl-cytosine, are found only in certain deoxyribose nucleic acids.



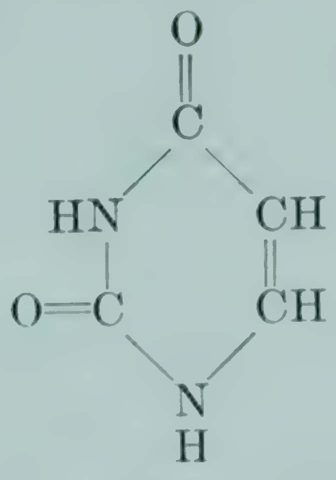
Adenine



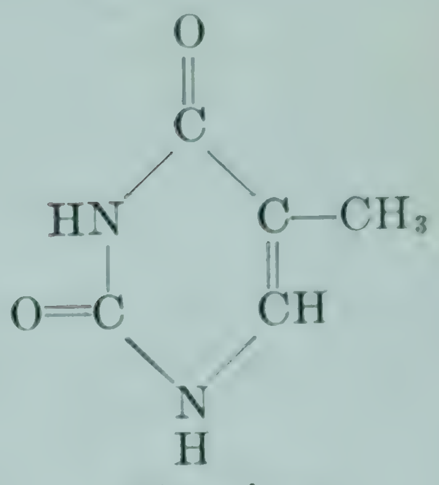
Guanine



Cytosine

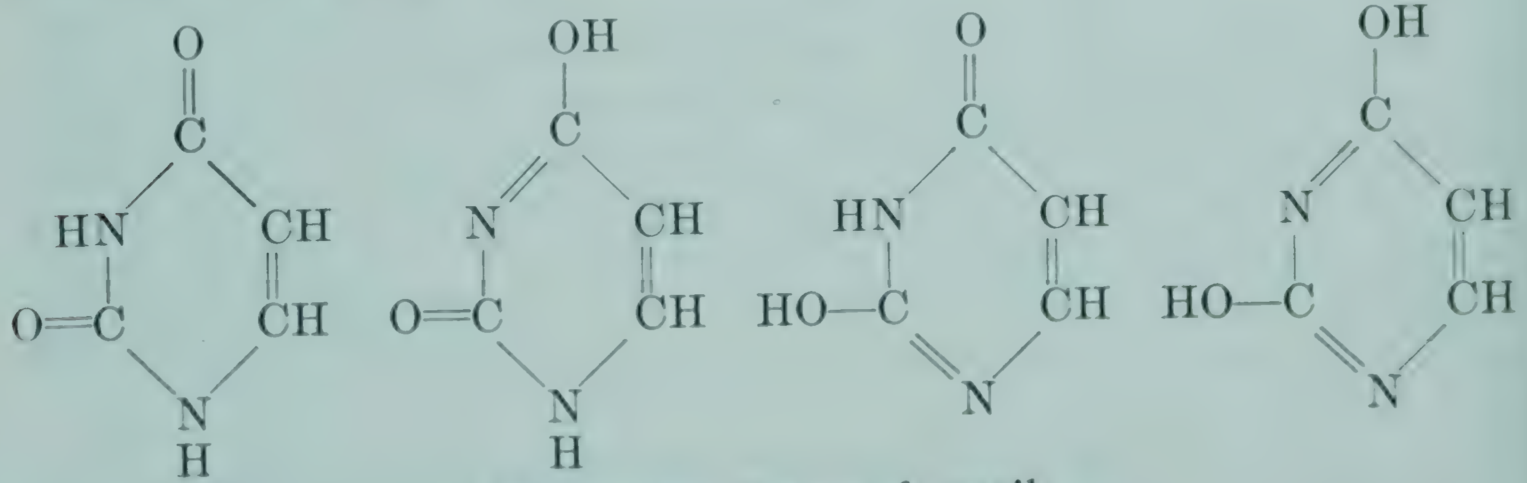


Uracil



Thymine
(5-methyl-uracil)

With all these structures, tautomerism from lactim to lactam forms is possible, for example:



Tautomeric forms of uracil

The Nucleotides and Nucleosides. Each purine or pyrimidine in a nucleic acid is found to be accompanied by one molecule of pentose and one of phosphoric acid. The studies of Levene, Jones, and others demonstrated that hydrolytic cleavage of the nucleic acids could lead to phosphoribosides, or *nucleotides*, of each of the bases. The nucleic acids are polymers of many such nucleotides, and hence the descriptive name *polynucleotides* is a useful one. If further hydrolysis is involved, the phosphoric acid is removed and the ribose derivatives of the bases, the *nucleosides*, are obtained. The relation between nucleosides and nucleotides may be indicated in the diagram and table shown on p. 203.

The nucleic acids used most extensively in the studies of their chemistry have been the ribose nucleic acid from yeast, or *yeast nucleic acid*, and the deoxyribose nucleic acid from thymus gland, or *thymus nucleic acid*. They contain approximately equimolar amounts of each of four bases. This led to a long prevalent theory that the nucleic acids were tetranucleotides, or at least contained a repeating tetranucleotide unit. However, with the advent of more refined techniques, analyses now indicate that few nucleic acids contain the equimolar quantities of the four bases required by the tetranucleotide theory. The presence of a fifth nucleotide in some deoxyribose nucleic acids also discredits that hypothesis. Present concepts of nucleic acid structure suggest a much greater complexity. These large molecules are still less well understood than are the proteins.

$$\overbrace{\underbrace{\begin{array}{l}\text{Purine or}\\ \text{Pyrimidine}\end{array} \text{\_\_\_\_} \begin{array}{l}\text{Ribose or}\\ \text{Deoxyribose}\end{array}}_{\textbf{Nucleoside}} \text{\_\_\_\_} \begin{array}{l}\text{Phosphoric}\\ \text{Acid}\end{array}}^{\textbf{Nucleotide}}$$

RIBOSE NUCLEIC ACID DERIVATIVES

Base	*Nucleoside*	*Nucleotide*
Uracil	Uridine	Uridylic Acid
Cytosine	Cytidine	Cytidylic Acid
Adenine	Adenosine	Adenylic Acid
Guanine	Guanosine	Guanylic Acid

DEOXYRIBOSE NUCLEIC ACID DERIVATIVES

Thymine	Thymidine	Thymidylic Acid

For the deoxyribose derivatives of the other three bases the prefix "deoxy-" is used with the names above, as in *deoxycytidine.*

Purine and Pyrimidine Content. Analyses of the purine and pyrimidine content of various deoxyribose nucleic acids by paper-chromatographic methods have shown variations in the composition of the DNA from various species. Chargaff has shown that though the ratio of total purines to total pyrimidines is usually close to unity, there are types in which the amounts of one purine and one pyrimidine may considerably exceed the amounts of the others. It is of interest that, of the various possible combinations, only two types have been encountered: one in which adenine and thymine predominate, and one in which guanine and cytosine predominate. The former is most frequently found in mammalian tissues, yeast and so forth; the latter is common in bacteria. Some bacteria possess a DNA with nearly equal proportions of the four bases. Where 5-methyl-cytosine is found to be present as a fifth component (in plant, fowl, and mammalian species, including man) it is usually found only in traces, although in the case of the DNA of wheat embryo it amounts to one-eighth of the total base present.

The Amount of Nucleic Acids in the Cells.

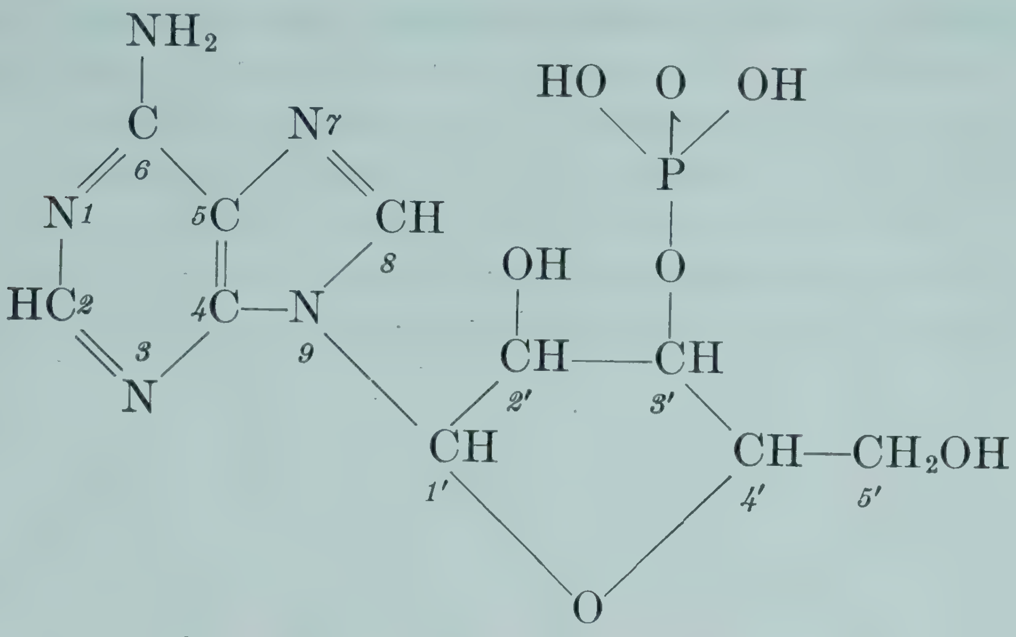
(a) DEOXYRIBOSE NUCLEIC ACID. The total quantity of deoxyribose nucleic acid per cell is remarkably constant in all the normal somatic tissues within a given species. This amount is not altered by conditions such as starvation or other stress. The amount per haploid (sperm) nucleus is one-half of that for a normal diploid nucleus of the species, and where polyploidy is found the DNA per nucleus is correspondingly increased. There are considerable variations from species to species, fish and birds having generally a smaller amount of DNA per nucleus than mammals (which generally contain in the order of 6×10^{-9} mg. per nucleus). The constancy of the amount of DNA per nucleus and of the composition throughout a species correlates well with the requirement of the genetic theory for a constancy in the character of the chromosome. This is another of the lines of evidence which foster the theory that DNA may be an integral component of the chromosome.

(b) RIBOSE NUCLEIC ACID. The ribose nucleic acids of various tissues vary in composition from tissue to tissue, and that of the nucleus differs from that of the cytoplasm. Preparations of RNA satisfactory for analytical studies are few, and no generalizations can be made. The quantity of RNA per cell fluctuates under various conditions. It is greatly influenced by such factors as the nutritional state of the animal and is greater in those cells with higher metabolic activities. For instance, it is high in secretory organs, such as salivary glands and pancreas, where active protein synthesis and secretion are taking place. There is also relatively more RNA in rapidly growing embryonic and tumor tissues. Much circumstantial evidence is available which indicates that there is an increase in quantity and metabolic activity of the RNA just prior to, or associated with, protein synthesis, and the theory that ribose nucleic acid plays a major role in the synthesis of protein has received much support. However, there is yet no concrete evidence of any direct participation of RNA in protein synthesis. Several other suggestions that RNA may be primarily involved in processes such as secretion or energy metabolism have some support. It remains that ribose nucleic acid is a dynamic component of the cell and is certainly associated with active metabolic processes; but a definite assignment of a functional role is not yet possible.

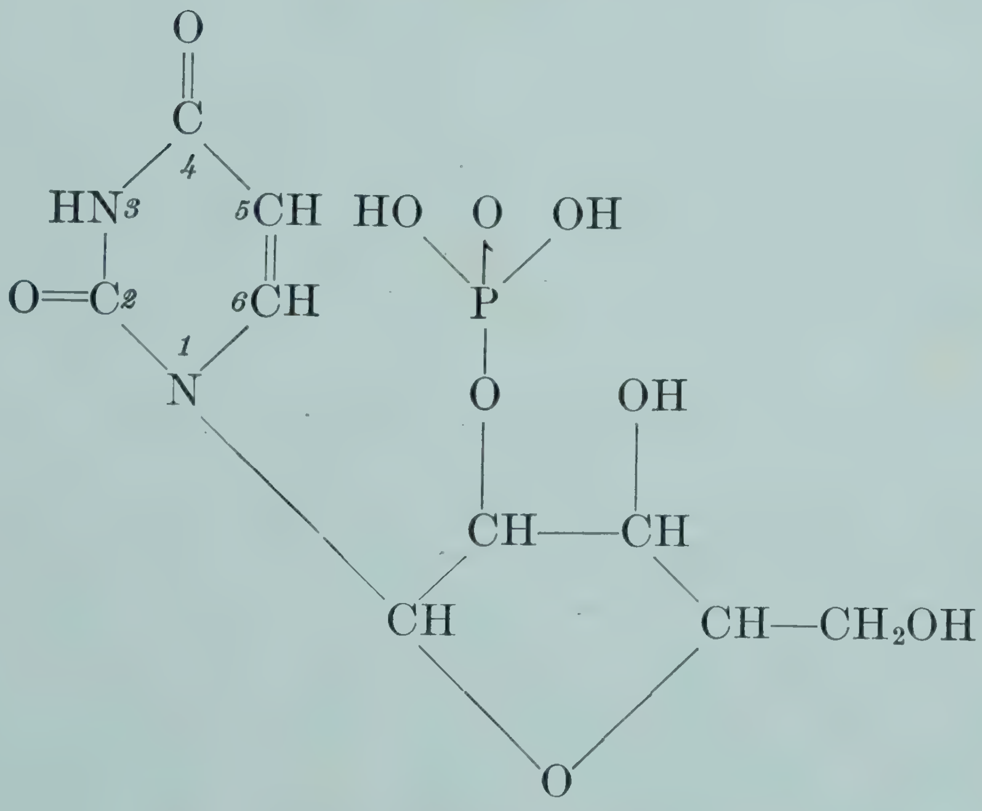
THE STRUCTURES OF NUCLEOTIDES AND OF POLYNUCLEOTIDES

The pentoses in the nucleosides are attached in a nonreducing N-glycosyl linkage. Note that the formation of a pyrimidine nucleoside requires that the 1 and 2 positions of the pyrimidine be in the lactam form. The positions of attachment, the existence in the furanose form, and the stereochemical configurations have been fully authenticated. In the case of the ribose nucleotides, three isomers are possible, depending upon which one of the hydroxyl groups of the sugar is esterified with the phosphoric acid. In the case of adenylic acid all of the three possible isomers are known (see p. 205).

Two isomers of each of the purine or pyrimidine nucleotides are produced upon alkaline hydrolysis of ribose nucleic acids. These are the 2′ and 3′ isomers, which were also known as the *a* and *b* isomers during a long uncertainty as to which was which. The chemical properties of the isomeric nucleotides are very similar, but it is possible to separate them by ion-exchange chromatographic techniques. The name *yeast adenylic* *acid* formerly referred to such a mixture of these isomeric adenylic acids, as obtained from yeast nucleic acid. The discovery that there were not four nucleotides, but four pairs of isomeric nucleotides that could be obtained from ribose nucleic acids, necessitated re-evaluation of many of the structural and metabolic aspects of the polynucleotides.



One of the isomeric Adenylic Acids
(9-β-D-ribofuranosyl-adenine-3′-phosphate)
(Adenosine-3′-phosphate)

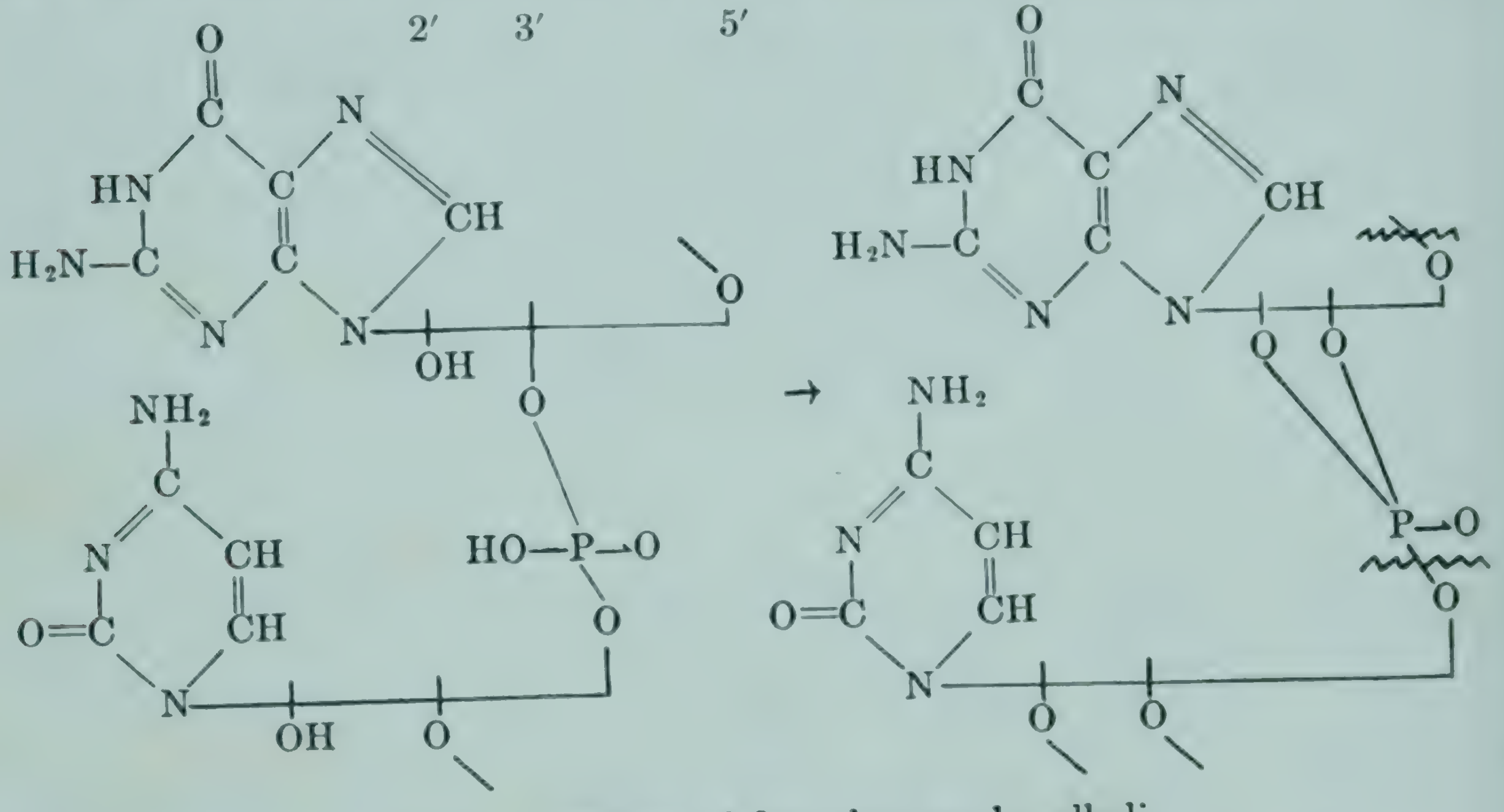


One of the isomeric Uridylic Acids
(1-β-D-ribofuranosyl-uracil-2′-phosphate)
(Uridine-2′-phosphate)

The third possible isomer of adenylic acid is the one in which the phosphate is attached at the 5′ position. This nucleotide, adenosine-5′-phosphate, has long been known to occur in muscle and bears the name *muscle adenylic acid*. It is the adenylic acid from which *adenosinetriphosphate* (ATP) and certain coenzymes are derived, and it thus plays important roles in carbohydrate metabolism, fermentation, and muscular contraction (see Chapter 10).

Enzymatic degradation has provided further information on the nature of the linkages present in RNA. Cleavage of RNA by pancreatic ribonuclease liberates the majority of the pyrimidines as the 3′-cytidylic and 3′-uridylic acids. An enzyme-resistant portion remains which is composed chiefly of purine nucleotides. The subsequent action of a phosphodiesterase (prepared from snake venom) leads to the production of all four 5′-mononucleotides, chiefly purine nucleotides but also small amounts of pyrimidine nucleotides. These results suggest that it is the 3′ and 5′ linkages which predominate in the nucleic acid, despite the fact that it is the 3′ and 2′ isomers that are produced by the action of alkali.

The production of the mixture of the 2′ and 3′ isomers upon alkaline hydrolysis of nucleic acids is attributed to the formation of cyclic phosphate esters, such as that depicted here. In this illustration the ribose and its three available hydroxyl groups are presented schematically:

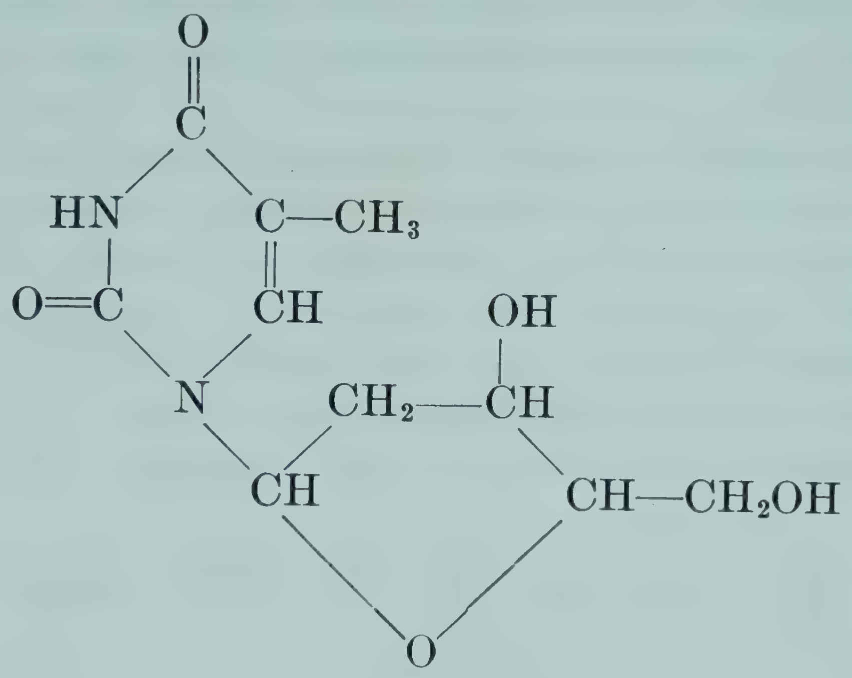


〰 **position of first cleavage by alkali**

In this triester of phosphoric acid the bond which is *not* involved in the cyclic phosphate is the most labile to alkali and is cleaved first. The cyclic mononucleotides which are thus liberated are then further cleaved by alkali to yield mixtures of the 2′ and 3′ mononucleotides.

In the deoxyribose nucleic acids the lack of a hydroxyl in the 2′ position of the sugar prevents the formation of the cyclic phosphate, as is evident from the formula of thymidine shown on p. 207. The deoxyribose nucleic acids are thus more resistant to depolymerization by alkali; in fact, most methods for separation of the two types of nucleic acid depend upon the difference in their stability in the presence of alkali.

In the ribose nucleic acid the presence of approximately one ionizable hydrogen per phosphate indicates that most of the phosphates are present as diesters. Approximately one quarter of this titratable hydrogen shows a pK of about 6, corresponding to a secondary phosphoryl dissociation, and this indicates that the ribose polynucleotides are not solely diphosphate ester bridges from the 3′ to the 5′ hydroxyls of adjacent nucleotides. Several other lines of evidence suggest the presence of some monoesterified ribose (i.e., oxidation by periodic acid, production of a dimethyl ribose after methylation), of some triesterified ribose (production of free ribose after methylation), and of some triesterified phosphoric acid (from titration data). The characterization of a number of di-, tri- and tetranucleotides, which are obtained by partial enzymatic or acid hydrolyses of ribonucleic acids, also reveals that several purine or pyrimidine nucleotides may be adjacent to one another. Sufficient information is not available to plot the sequence of nucleotides in any nucleic acid.



Thymidine

(1-β-D-2-deoxyribofuranosyl-thymine)

A formulation such as that shown in Fig. 59 is compatible with the present chemical and enzymatic evidence and indicates the complexity of the structure of the polynucleotides known as *ribose nucleic acids*. This

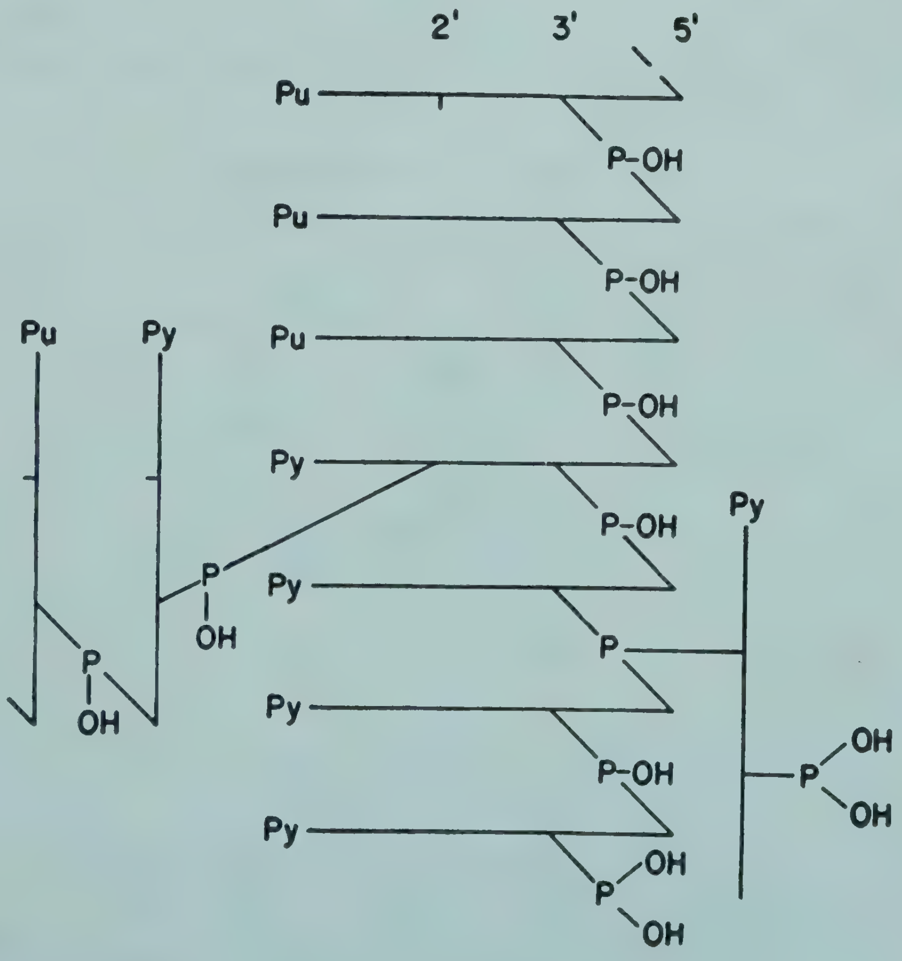


FIG. 59. FRAGMENT OF A RIBOSE NUCLEIC ACID.

Schematic presentation of the types of linkage involved.

(After Cohn, Doherty, and Volkin: *Phosphorus Metabolism*, Vol. 2, Baltimore, Johns Hopkins University Press, 1952.

sketch of the current speculations regarding intermediate linkages represents only a portion of a molecule. A molecular weight of 30,000, for instance, requires nearly 100 nucleotides.

Various enzymatic hydrolyses of the deoxyribose nucleic acids have yielded 3′-nucleotides or 5′-nucleotides. Small amounts of deoxyribose nucleosides bearing two phosphoric acids (in both the 3′ *and* 5′ positions) have also been obtained. The nucleotides in DNA must be joined principally by 3′, 5′ phosphate diester bridges, with possibly some branching through triesterified phosphate.

It should be pointed out that phosphate bridges between the 5′ hydroxyls of two nucleotides have not been found, nor have pyrophosphate linkages been implicated. The latter type of linkage is found in those coenzymes which are formed from adenosine-5′-phosphate and various vitamin derivatives. Despite their considerable similarities, there is thus a fundamental difference between nucleoproteins and those enzymes which are complexes of a dinucleotide coenzyme and a protein (see Chapter 12).

METABOLISM OF NUCLEIC ACIDS

The organism is able to synthesize *de novo* all of its nucleic acids and none of the organic constituents thereof are necessary in the diet. It has therefore been difficult to learn what compounds are utilized in forming the tissue nucleic acids, since classical nutritional and balance studies were able to contribute little. Most of the knowledge of the origin of the nucleic acid components has come from the administration of isotopically labeled compounds and the use of degradative procedures which permit analysis of individual moieties of the products. In this way the principle sources of the individual atoms of the purine ring have been shown to be:

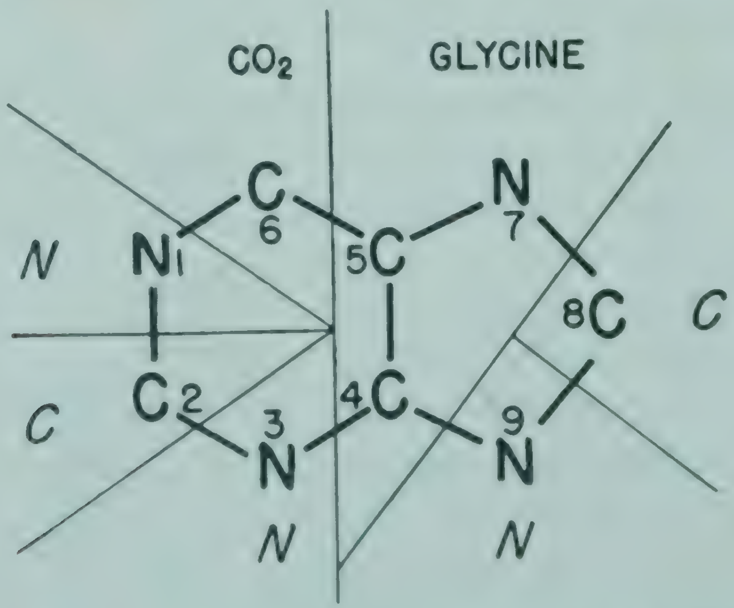


FIG. 60. ORIGIN OF THE ATOMS OF THE PURINE RING.

C—from 1-carbon sources (formate, formaldehyde); *N*—from NH_3 (via: aspartate, glutamate, glutamine).

From Brown, Roll, and Weinfeld: *Phosphorus Metabolism*, Vol. 2, Baltimore, Johns Hopkins University Press, 1952.

The 4,5-carbon and 7-nitrogen portion is derived from glycine as a single unit; the 6 carbon arises from carbon dioxide. The 2 and 8 carbons arise from one-carbon units such as formate or formaldehyde, and folic acid plays a direct role in the incorporation of these one-carbon units. There is no evidence that there is any formation of free purines in the course of biosynthesis in tissues. Rather, the ribose and phosphate are attached to a smaller precursor before the synthesis of the purine ring is fully completed. This has been shown to be true in studies of inosinic acid (hypoxanthine ribotide) biosynthesis in pigeon liver, and the mechanism of synthesis of purine derivatives in this species is probably identical or very similar to such synthesis in the mammal.

In birds and reptiles there is a peculiarity of purine metabolism not observed in mammals. In those species uric acid, instead of urea, is formed directly as the chief catabolic end product of protein nitrogen metabolism.

Among the purines classically associated with purine metabolism, only adenine reaches the nucleic acids in significant amounts when fed to the rat. Adenine is not only incorporated as adenine but is also partially transformed into the guanine of the polynucleotides; and by the use of isotopes it has been demonstrated that the purine ring remains intact during this conversion. Guanine and several other purines are extensively catabolized and, in the rat, do not reach the polynucleotides. Guanine can, however, be readily used by several species of microorganisms, and to a small extent by the mouse, and in most cases it can also be transformed into polynucleotide adenine. A purine which has not been shown to occur in nature, 2,6-diaminopurine (or 2-aminoadenine), can be utilized for the synthesis of polynucleotide purines. In the rat it is converted only into nucleic acid guanine, although in some species it may be converted into both nucleic acid purines.

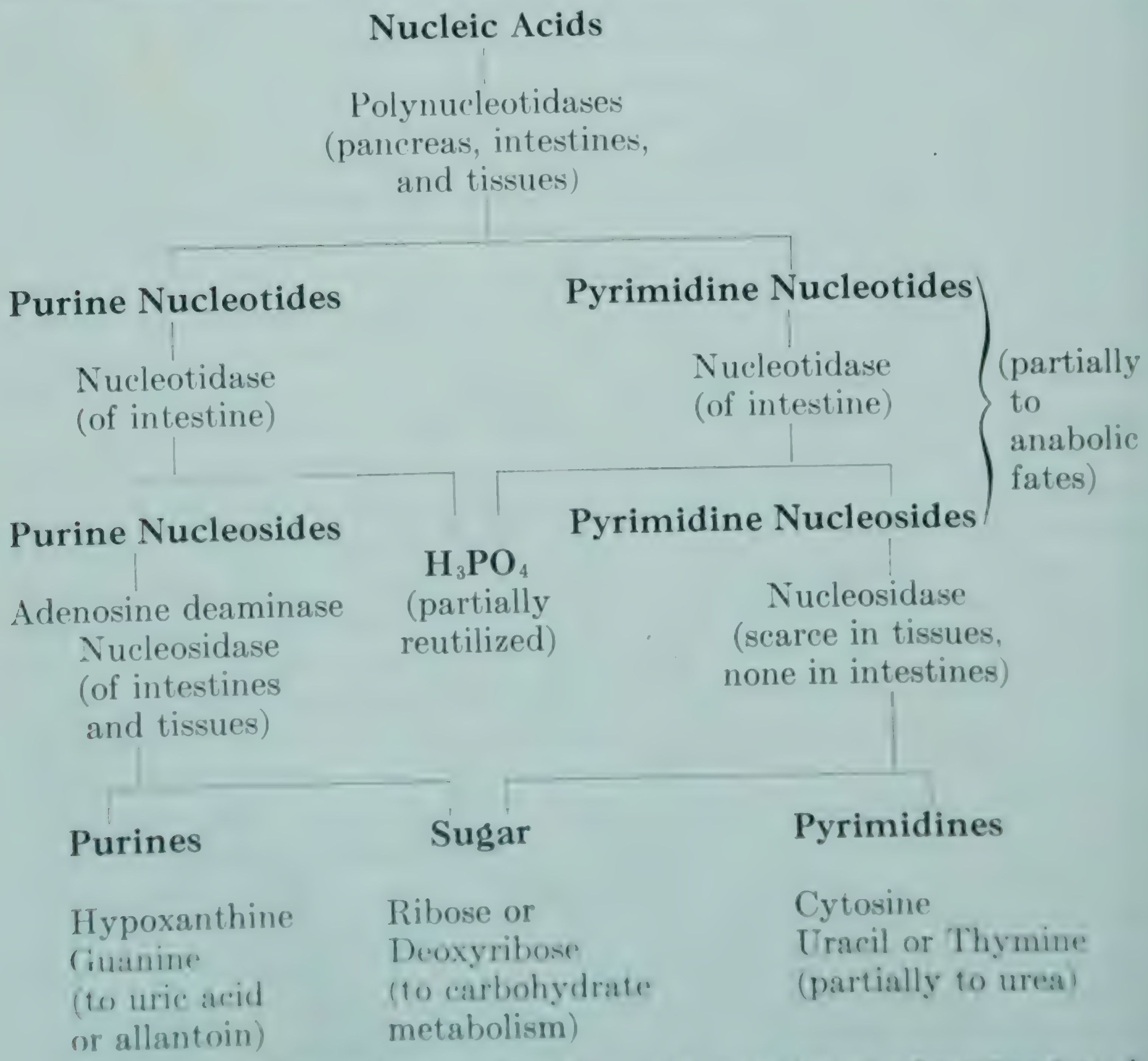
The nucleoside and nucleotides of adenine may be utilized by the rat, but only if they are administered parenterally, in order to avoid the digestive enzymes of the intestinal tract. Guanylic acid can lead to nucleic acid guanine, although guanosine, like guanine, fails to do so in the rat.

Less is known of the origin of pyrimidines. The 2 carbon can arise from carbon dioxide; and aspartic acid and carbamyl aspartic acid are major precursors of the other carbons of the ring. The pyrimidines uracil and cytosine are not significantly utilized for polynucleotide synthesis, although their nucleosides or nucleotides are very effectively utilized. There is an interconversion of the pyrimidine derivatives, similar to that of the purines, and thus cytidine and cytidylic acid are transformed into ribose nucleic acid uracil and also into deoxyribose nucleic acid cytosine and thymine. The conversion to the latter involves the acquisition of an additional carbon for the 5-methyl group of the thymine. This arises from one-carbon units such as formic acid or the β-carbon of serine; and folic acid is also involved in this reaction. Vitamin B_{12} is likewise involved in the formation of thymidine and other deoxyribosides, but its role is not yet adequately defined. It is of interest that a pyrimidine which is found in milk, orotic acid, or 6-carboxyuracil is readily utilized as a precursor of polynucleotide pyrimidines. Its possible significance in infant nutrition is not clarified.

The usual diet contains few sources of individual purines, pyrimidines, and their nucleosides or nucleotides. Varying amounts, however, of nucleic acids will normally be encountered in the diet, particularly when appreciable quantities of glandular meats are consumed. The feeding of isotopically labeled ribose (yeast) nucleic acid to the rat showed that there was appreciable incorporation of its pyrimidines, but no significant incorporation of its purines, into the tissue nucleic acids. This is in distinct contrast to the fact that when the individual free pyrimidines are present in the diet they are not incorporated. Since adenine and its derivatives can be incorporated it is obvious that the purine moieties of the nucleic acid are catabolized further than the nucleotide or nucleoside stage, and that the catabolic products do not include adenine. On the other hand the pyrimidine moieties which were utilized must have been absorbed before degradation to the free pyrimidines. The fate of the dietary nucleic acid can be correlated with the known fates of the various derivatives and with the known degradative enzymes described below.

ENZYMATIC BREAKDOWN OF NUCLEIC ACIDS

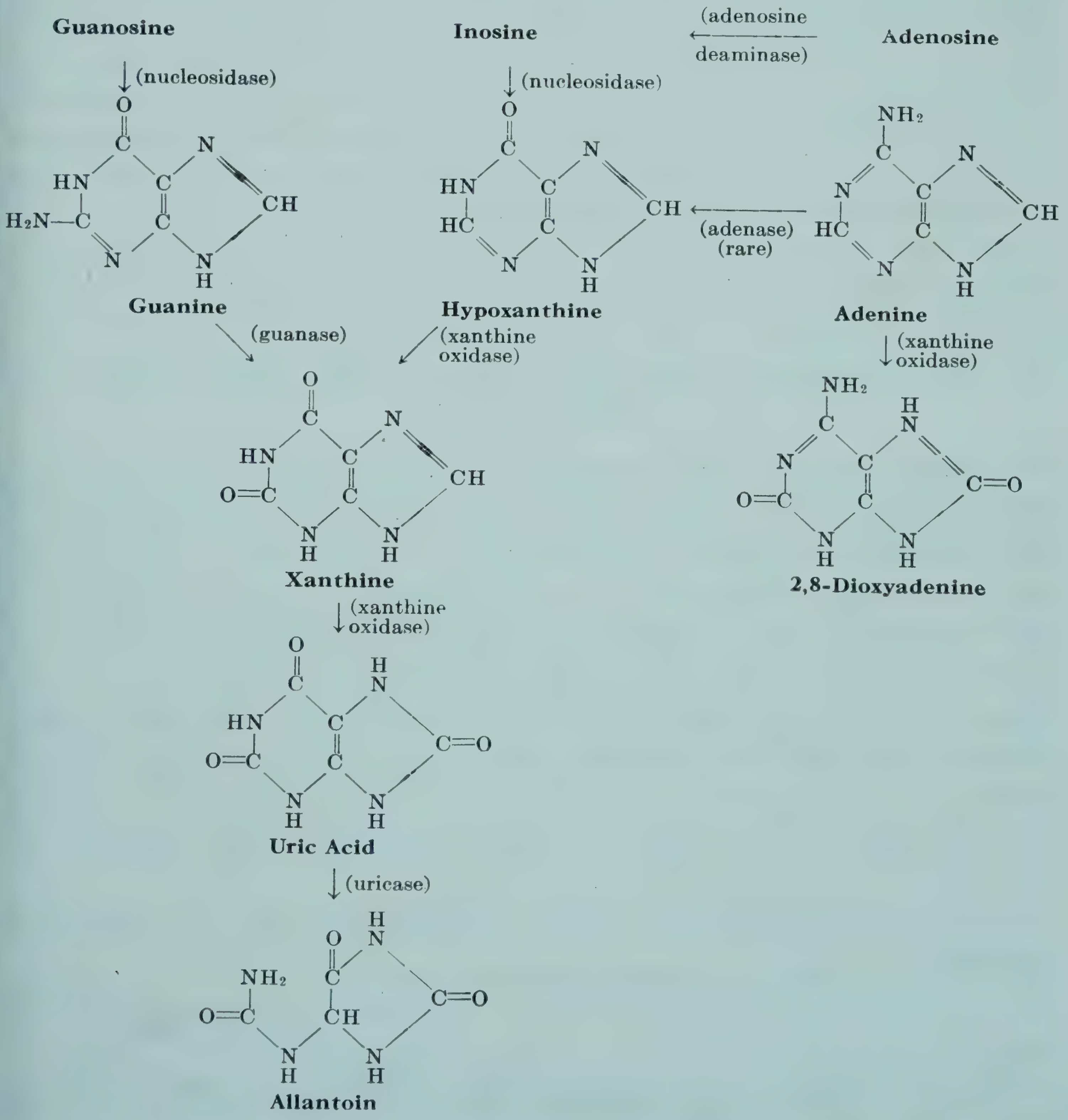
The following is an outline of the steps and the enzymes involved in the digestion and catabolism of nucleic acids:


Nucleic Acids
Polynucleotidases
(pancreas, intestines,
and tissues)
Purine Nucleotides
Pyrimidine Nucleotides
Nucleotidase
(of intestine)
Nucleotidase
(of intestine)
(partially
to
anabolic
fates)
Purine Nucleosides
H_3PO_4
(partially
reutilized)
Pyrimidine Nucleosides
Adenosine deaminase
Nucleosidase
(of intestines
and tissues)
Nucleosidase
(scarce in tissues,
none in intestines)
Purines
Sugar
Pyrimidines
Hypoxanthine
Guanine
(to uric acid
or allantoin)
Ribose or
Deoxyribose
(to carbohydrate
metabolism)
Cytosine
Uracil or Thymine
(partially to urea)

The known mammalian nucleosidases do not cleave adenosine but do split inosine (hypoxanthine riboside) and guanosine. The very active adenosine deaminase does, however, convert adenosine to inosine, and the net result is to lead to purines which are extensively catabolized and not to purines which are utilized for nucleic acid synthesis. The lesser abundance of pyrimidine nucleosidases does not result in the rapid destruction of the ribose derivatives of the pyrimidines.

CATABOLISM OF PURINES

The purines are readily catabolized to uric acid via a series of deaminations and oxidations as outlined below. Adenosine and its derivatives can reach hypoxanthine through deamination to inosine and cleavage of the inosine to hypoxanthine. If a large amount of adenine is present it can be oxidized directly to 2,8-dioxyadenine, which may cause kidney damage through deposition of crystals of this very insoluble substance in the tubules.


Guanosine
(nucleosidase)
Guanine
Inosine
(adenosine deaminase)
Adenosine
(nucleosidase)
Hypoxanthine
(adenase) (rare)
Adenine
(guanase)
(xanthine oxidase)
(xanthine oxidase)
Xanthine
2,8-Dioxyadenine
(xanthine oxidase)
Uric Acid
(uricase)
Allantoin

In man and apes, uric acid is the primary end product of purine metabolism, but in other mammals an enzyme, uricase, carries the oxidation further to the more soluble allantoin. In man intravenously administered uric acid is almost completely excreted as such, and only a small but variable amount is degraded as far as urea. However, orally administered uric acid is extensively degraded to urea, presumably by intestinal bacteria.

In gout the blood uric acid concentrations are increased somewhat above normal, and the ultimate result of the disease is the deposition of tophi of sodium urate in the joints and elsewhere. However the uric acid clearance is usually essentially normal, although it may be decreased where renal impairment has resulted.

In the normal individual there is somewhat less than a gram of uric acid in the blood and fluids, in a volume approximately equal to the extracellular space. The size of this available pool, and its rate of renewal, can be measured by the intravenous injection of a small sample of isotopic uric acid and determination of the course of excretion of the isotope. Benedict and Stettin have shown that in the gouty individual the size of the pool may be increased many fold. Only the outer portions of the tophaceous deposits of urates were found to equilibrate rapidly with administered isotopically labeled uric acid. The etiology of gout is complex but a study making use of isotopic glycine has confirmed the view that increased synthesis of uric acid is a factor.

The administration of ACTH leads to uricaciduria, and in the gouty patient can precipitate an attack of acute gouty arthritis. Increased blood uric acid is one of the outstanding changes observed in eclampsia.

EXPERIMENTS ON NUCLEOPROTEINS AND THEIR DERIVATIVES

1. *Preparation*[1] *of Deoxyribosenucleoprotein from Thymus or Spleen.* Fresh tissue is minced and thoroughly washed with physiological saline, containing 0.01 M sodium citrate. The washed tissue is then extracted by stirring with about 10 volumes of 1 M NaCl containing 0.01 M sodium citrate. The suspension is centrifuged at high speed (10,000 to 12,000 r.p.m.) and the supernatant liquid containing the nucleoprotein is removed. This solution is poured into 6 volumes of water (which reduces the NaCl concentration to that of an isotonic solution), and the nucleoprotein precipitates as a fibrous mass. The supernatant liquid is decanted and the nucleoprotein is purified by again dissolving in M NaCl. The solution is centrifuged at high speed to remove any suspended material, and the nucleoprotein is again precipitated by pouring into 6 volumes of water. If the mixture is stirred with a rod having a crook at its end, the fibrous material generally winds around the rod and adheres to it.

2. *Tests on Nucleoprotein.* Try the following tests on a nucleoprotein preparation.

a. Try the xanthoproteic, Millon, and biuret tests.

b. Test the solubility in water, 10 per cent NaCl, 10 per cent HCl, dilute KOH, and alcohol.

c. Test for Phosphorus in Organic Matter. To a small amount of the substance in a crucible add about five times its bulk of fusion mixture (two parts sodium carbonate to one potassium nitrate). Heat carefully until the resulting mixture is colorless. Cool, dissolve the mass in a little warm water, acidify with nitric acid, heat to about 65°C., and add a few ml. of molybdate solution. In the presence of phosphorus a yellow precipitate of ammonium phosphomolybdate is formed.

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[1] Mirsky and Pollister: *J. Gen. Physiol.*, 30, 117 (1946); Petermann and Lamb: *J. Biol. Chem.*, 176, 685 (1948).

Instead of acidifying with nitric acid, the aqueous solution may be approximately neutralized with hydrochloric acid, a few ml. of magnesia mixture added, and then excess of ammonium hydroxide solution. A white precipitate of magnesium ammonium phosphate is formed.

d. Dissolve a little of the nucleoprotein in very dilute KOH and then make slightly acid with acetic acid. Explain results.

3. *To Show the Presence of Protein, Carbohydrate, Phosphoric Acid, and Purine Radicals in Nucleoprotein.* Upon complete acid hydrolysis of nucleoprotein, these substances will be liberated, as well as decomposition products of the protein part of the molecule. To show their presence, proceed as follows: Transfer about a gram of nucleoprotein to a small flask and add 50 ml. of 5 per cent H_2SO_4. Boil for an hour or more to hydrolyze. Maintain the original volume by adding water. The solution becomes brown owing to the formation of melaninlike substances. Cool and filter the acid solution. Apply the following tests to portions of it: (a) the biuret test, (b) the xanthoproteic test, (c) the Molisch test, (d) Benedict's test, and (e) the test for phosphate. (f) Transfer about 25 ml. of the hydrolyzate to a casserole and add ammonia with thorough mixing, a little at a time, until the fluid is nearly neutral. Then make slightly alkaline with dilute ammonia and filter if not clear. Transfer to a beaker and add about 10 ml. of 5 per cent ammoniacal silver nitrate solution. Purine bases will yield a flocculent precipitate of their silver salts. If a precipitate does not appear immediately, examine the solution after it has been allowed to stand undisturbed for some time.

4. *Preparation of Ribose Nucleic Acid from Yeast.* Dilute 50 ml. of 1 per cent NaOH with 250 ml. of water in a casserole and add to this solution 100 g. of compressed yeast cut in small pieces or 30 g. of dry yeast. Heat on the water bath for half an hour with occasional stirring. Remove from the bath and filter at once through a folded filter. To the cooled filtrate add acetic acid until faintly acid to litmus. Filter again. Evaporate the solution to 100 ml. or less, and filter if necessary. Allow to cool to 40°C. or below, then pour with vigorous stirring into 200 ml. of 95 per cent alcohol containing 2 ml. of concentrated HCl. Allow to settle in a tall vessel and decant. Wash twice with 95 per cent alcohol and twice with ether. Transfer to a filter paper. Allow to drain and dry at room temperature.

5. *Tests on Nucleic Acid from Yeast.*[2]

a. Test the solubility of nucleic acid in cold and hot water, in alcohol, and in dilute acid and alkali. To the solution in alkali add dilute HCl drop by drop until the solution is acid; then add excess of concentrated HCl.

Does nucleic acid coagulate on boiling? Does the solution in hot water gelatinize on cooling?

b. Try the biuret test.

c. Dissolve a little nucleic acid in water with the aid of heat. Test the reaction of different portions of the solution with litmus, alizarin, and Congo red solution.

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[2] A satisfactory preparation of yeast nucleic acid may be obtained from Schwarz Laboratories, Inc., Mount Vernon, New York.

d. Boil a small portion of the nucleic acid with about 10 ml. of 10 per cent sulfuric acid for one to two minutes. Divide into three portions.

To one portion apply carbohydrate tests—e.g., the α-naphthol (Molisch) reaction and Tauber's test. What do these indicate?

To a second portion apply a test for purine bases. Add an excess of ammonia and then a little silver nitrate solution.

To the third portion apply the test for phosphate, adding ammonia in slight excess, then making acid with nitric acid, adding molybdate solution, and warming.

6. *Preparation of Deoxyribose Nucleic Acid.* Freshly excised thymus glands are minced and frozen. The subsequent extractions are carried out as near 0°C. as possible. Homogenize 100 g. of frozen tissue in 200 ml. of cold 0.15 M NaCl solution, which is also 0.01 M in sodium citrate, and centrifuge the mixture at 2000 r.p.m. This operation is repeated three times on the residue and the supernatants are discarded.

The residue is dispersed in about 200 ml. of water[3] in a high-speed blendor. With continued stirring sufficient 5 per cent sodium dodecyl sulfate is added to bring the concentration to 0.5 per cent, and sufficient NaCl to make the solution 1 M with respect to it. The mixture is stored at 5°C. overnight and then filtered through a pad of Celite on a Buchner funnel. When clear, the filtrate is poured, with stirring, into two volumes of ethanol. The fibrous precipitate of sodium deoxyribose nucleate is collected and is washed repeatedly with 70 to 95 per cent alcohol.

7. *Tests on Deoxyribosenucleic Acid.* Repeat Exps. 5 (a) through (d) as given under yeast nucleic acid, above. (e) Make a 4 per cent solution of deoxyribosenucleic acid in hot water (0.4 g. to 10 ml.). Allow to cool. What happens? Divide into two portions. To one add a little NaOH solution; to the other add acetic acid. Then neutralize carefully in each case.

Both acetic acid and NaOH decrease the viscosity of the nucleate solution. It may be changed back and forth from the gelatinous to the fluid condition by the alternate addition of acid and alkali.

8. *Tests on Purine and Pyrimidine Bases and Derivatives.*

(a) XANTHINE.

(1) *Silver Nitrate Reaction.* Dissolve a little xanthine in ammonia and add silver nitrate solution. Examine a little of the precipitate microscopically (see Fig. 61).

(2) *Copper Sulfate Reaction.* Dissolve a little of the substance in dilute alkali, make faintly acid with acetic acid. Heat to boiling. Add 1 ml. of 10 per cent $CuSO_4$, and then a few drops at a time of sodium bisulfite (saturated solution) until the precipitate becomes yellowish. All of the purines give this reaction.

(3) *Nitric Acid Test.* Place a small amount of the substance in a small evaporating dish, add a few drops of concentrated nitric acid, and evaporate to dryness very carefully on a water bath. The yellow residue upon moistening with caustic potash becomes red in color and upon further heating assumes a purplish-red hue. Now add a few drops of water and warm. A yellow solution results which yields a red residue

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[3] For best results the solution should be analyzed for nitrogen and the nitrogen concentration adjusted to 0.5 mg. per ml. Marko and Butler: *J. Biol. Chem.*, **190**, 165 (1951).

upon evaporation. Compare with a similar reaction on other purine bases and uric acid. (See the murexide test, p. 796.)

(b) HYPOXANTHINE.

(1) Repeat Exps. 1 and 3 under Xanthine. Examine the crystals of hypoxanthine silver nitrate under the microscope. (See Fig. 62.)

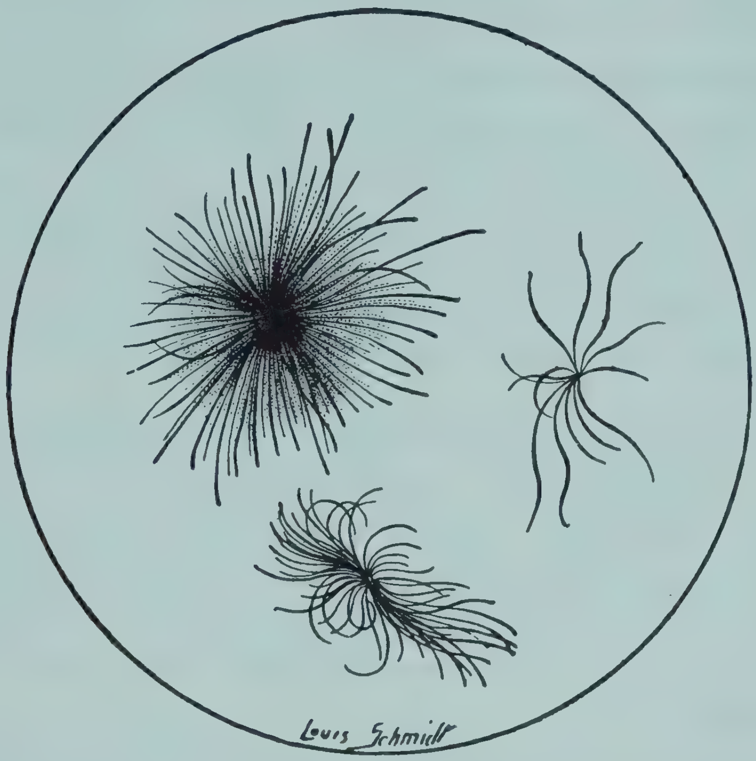

FIG. 61. XANTHINE SILVER NITRATE.

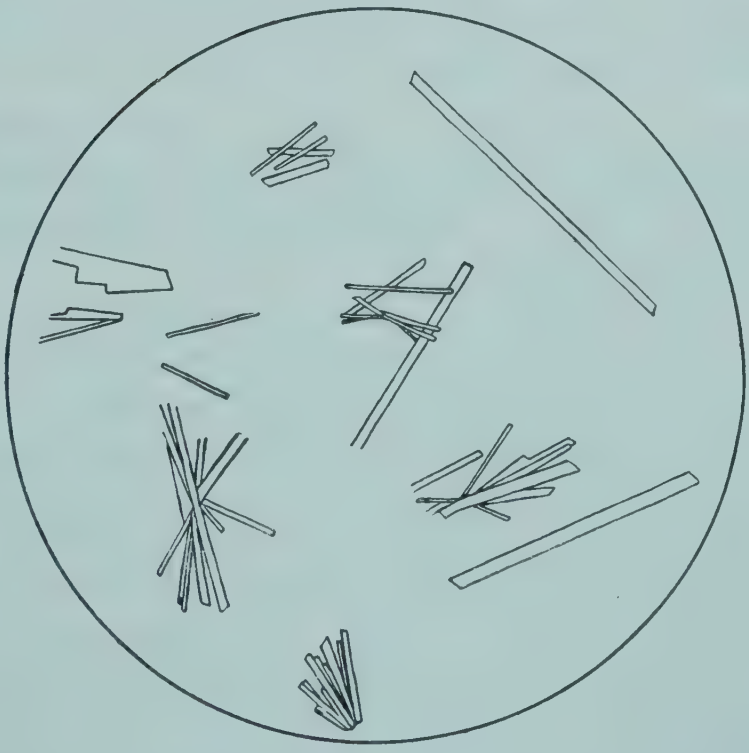

FIG. 62. HYPOXANTHINE SILVER NITRATE.
Drawn from a student preparation by Dr. E. F. Hirsch.

(2) Dissolve a little of the substance in a very small amount of hot 6 per cent nitric acid and allow to cool. Characteristic whetstone crystals of hypoxanthine nitrate should be formed. Examine under the microscope.

(c) ADENINE.

(1) Dissolve a little adenine in hot water and add a few drops of picric acid. Examine the pale yellow clusters of needles under the microscope.

(2) Repeat Exp. 3 under Xanthine.

(d) GUANINE.

(1) Dissolve a little of the substance in 20 to 25 times its weight of boiling 5 per cent alcohol. Allow to cool and examine the crystals under the microscope.

(2) Dissolve a little guanine in 20 to 25 times its weight of boiling 5 per cent hydrochloric acid. Allow to cool, and examine the crystals.

(3) Perform Exp. 3 under Xanthine.

(e) URIC ACID. On a small amount of uric acid, try Test 3 as given under Xanthine. This test on uric acid is called the murexide test. For other tests on uric acid see Chapter 28, Urine.

(f) REACTIONS FOR URACIL, CYTOSINE, AND THYMINE. Treat an aqueous solution of each pyrimidine with an excess of bromine. Remove the excess by boiling the solution. Add barium hydroxide in excess. A purple color indicates cytosine or uracil and is due to the purple barium salt of dialuric acid.

Reflux for a few minutes and distill. If thymine is present, CO_2, urea, and acetol (CH_3COCH_2OH) are formed, and the acetol distills over. Make strongly alkaline with NaOH. Add several drops of *o*-aminobenzaldehyde.[4] Evaporate over a flame to half the volume. Cool. Make distinctly acid with HCl and then alkaline with $NaHCO_3$. Filter. A blue fluorescence of 3-oxyquinaldine indicates acetol and the original presence of thymine.

(g) ULTRAVIOLET ABSORPTION SPECTRA. The availability of convenient and relatively inexpensive ultraviolet spectrometers has made it possible to make most of the quantitative and qualitative measurements of the individual purines and pyrimidines through their strong and characteristic ultraviolet absorption spectra. See also p. 283.

9. *Methods for the Separation of Purines and Pyrimidines.* Procedures which involve fractional crystallization for the separation of purines and pyrimidines have been largely superseded by the techniques of ion-exchange separation or paper chromatography.

(a) CRYSTALLIZATION OF DERIVATIVES OF GUANINE AND ADENINE FROM NUCLEIC ACID. Place 5 g. of yeast nucleic acid in a 200-ml. Erlenmeyer flask, add 100 ml. of boiling-hot 5 per cent sulfuric acid and heat carefully over a small flame to bring the nucleic acid into solution. During this heating, which lasts only a minute, the contents of the flask should be kept constantly in motion to avoid charring the undissolved nucleic acid. Close the flask with a cork bored with one hole into which is inserted a condensing tube. Immerse in a boiling water bath and heat for about an hour. Disconnect the flask and while its contents are still hot add concentrated ammonia a drop at a time until the fluid is slightly alkaline, and then add 5 ml. of concentrated ammonia in excess. Guanine is thus precipitated in granular form, while all of the other products including phosphoric acid and adenine remain in the ammoniacal solution.

After several hours, collect the precipitated guanine and wash it with 1 per cent ammonia. Dissolve in as small an amount of 20 per cent sulfuric acid as possible, add a little charcoal, and boil. Filter, heat to boiling, and precipitate with an excess of ammonia. Filter, dry the precipitate at 40°C., and dissolve it in about 20 parts of boiling 5 per cent hydrochloric acid. As the solution cools, guanine hydrochloride separates as needle-shaped crystals.

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[4] Mix 3 g. of crystalline *o*-nitrobenzaldehyde with 50 g. of crystalline ferrous sulfate. Add 75 ml. of concentrated ammonia. Heat on a steam bath for one hour. Distill off the *o*-aminobenzaldehyde with steam. The mixture before distillation will keep for two weeks.

Combine the ammoniacal filtrates obtained in the isolation and purification of guanine. Filter if necessary. The ammonia is then boiled off and an excess of picric acid added. The yellow precipitate of adenine picrate is collected and dried. The adenine picrate can be recrystallized from 25 per cent acetic acid. Adenine can be recovered from the picrate by acidification with HCl and long extraction with ether, or by the use of an ion-exchange resin which will take up the picric acid.

(b) SEPARATION OF PURINES BY ION-EXCHANGE CHROMATOGRAPHY. Place a loose pad of glass wool in the bottom of a 50 ml. burette. Add about 25 ml. of water and then sufficient suspension[5] of Dowex 50 (hydrogen ion form) to make a resin bed 2 cm. high. Wash the resin into place by filling the buret to the top with water and allowing it to drain. Use a long rod to push another loose pad of glass wool down to the top of the resin bed. The rate of flow should be about 7 or 8 ml. per minute when the buret is full, and 2 or 3 ml. per minute for the last few ml. in the buret. The stopcock should always be closed just before the liquid level reaches the top of the resin bed. Arrange a series of eight numbered tubes for collection of fractions from the column.

Charge the column by pouring in a solution of 0.5 mg. of hypoxanthine and 0.5 mg. of adenine in 50 ml. of 0.1 N HCl and allowing it to drain. Wash the column with a few ml. of water and combine the effluents as Fraction 1. Elute the purines from the column with five consecutive 25-ml. portions of 2 N HCl. Then elute with two 25-ml. portions of 4 N HCl for Fractions 7 and 8.

Test for purines in the eluates by reduction, diazotization of the products of reduction, and coupling with the Bratton-Marshall reagent, as follows. Place about 5 ml. of each fraction in clean test tubes. To equalize the acid concentrations add 2 ml. of 6 N HCl to Fraction 1, and dilute Fractions 7 and 8 with equal volumes of water. As controls use 0.025 mg. of hypoxanthine and 0.025 mg. of adenine, each in 2 N HCl, and a 2 N HCl blank. 1) To each tube add a pinch of Zn dust the size of a match head and place immediately in a boiling water bath for 5 to 7 minutes. 2) Cool the tubes by transferring to a beaker of tap water and filter each. 3) To each filtrate add 1 ml. of 0.3 per cent sodium nitrite solution. 4) After 10 minutes add 1.5 ml. of 0.5 per cent ammonium sulfamate solution. 5) Add to each 1 ml. of 0.2 per cent N-(1-naphthyl)ethylenediamine solution.[6] The bulk of the hypoxanthine (which leads to a pink-purple color) should be in Fractions 2 and 3. A little adenine (which yields an orange-pink) will appear in Fraction 6, but most of it in Fraction 7. The pattern of elution from the column depends to a considerable extent upon the length and compactness of the resin bed.

With larger columns operated more slowly, it is possible to effect more complete separation of complex mixtures of purine and pyrimidine derivatives and of isomeric nucleotides, than by the foregoing method. For a discussion of ion-exchange resins and column chromatography, see Chapter 1.

(c) PAPER CHROMATOGRAPHIC SEPARATIONS OF PURINE AND PYRIMIDINE DERIVATIVES. Preparation of the paper: On a sheet of filter paper 17 by 26 cm. (Schleicher and Schull No. 597) draw a light pencil line 2 cm. from one of the long edges. Place single drops of the compounds to be studied at intervals of 2 or 3 cm.

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[5] Dowex 50 (H^+) 200–400 mesh, is prepared by suspension in 5 volumes of water and removal, by decantation, of any very fine material which settles slowly. The resin is then washed by heating 1 hour in 2 N NaOH on a steam bath; then with 4 or 5 changes of 4N HCl (or in a column until free of material absorbing in the ultraviolet). It is then washed by repeated changes of water until the effluent is nearly neutral. Store under 2 volumes of distilled water. When well shaken, 6 ml. of the suspension will furnish 2 ml. of resin.

[6] This solution is stored in a dark bottle, and all solutions for the Bratton-Marshall test should be prepared fresh each week.

along this line and allow them to dry thoroughly (or dry under an infrared lamp). If more than one drop proves to be needed, the first must be thoroughly dry before the second is added. Each spot should contain about 25 μg. Roll the paper and staple it to form a cylinder 17 cm. high (do not overlap the edges of the paper but allow the staples to serve as links between the edges).

Stand the cylinder of paper in a 10 cm. petri dish filled to a depth of about 5 mm. with the appropriate solvent. Cover with a bell or battery jar.[7] When the solvent has migrated nearly to the top edge of the paper, flatten the paper and dry, in a well-ventilated oven if possible.

Inspect the papers under a low-intensity ultraviolet lamp[8] and outline in pencil those dark areas where the compounds absorb or quench the fluorescence of the paper. The distance the compound moves, expressed as a fraction of the distance between the starting point and the solvent front, is known as the R_F value of the compound. For a discussion of paper chromatography, see Chapter 1.

1) Spot adenine, guanine, hypoxanthine, and mixtures of adenine and guanine and of adenine and hypoxanthine. Develop with *n*-butanol saturated with water, with a small container containing a few ml. of conc. NH_4OH also placed under the jar. Allow about 3 to 4 hours for development.

2) Spot the same compounds and develop 3 hours in 70 per cent ethanol. This solvent is rapid, but the spots are less discrete. Adenine and hypoxanthine can be differentiated from guanine, but not from each other.

3) Spot guanine, adenine, cytosine, uracil, thymine, and mixtures of guanine and uracil and of adenine and thymine. Develop in isopropanol-2 N HCl,[9] for about 16 hours. This solvent moves slowly but does permit differentiation of all these five bases.

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Miescher: *Die Histochemischen und Physiologischen Arbeiten*, Leipzig, Vogel, 1897.

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[7] Preferably with a tightly fitting glass plate as a base.

[8] Mineralight, V-44, Ultraviolet Products, Los Angeles.

[9] This solvent must be prepared fresh each day. To 16.4 ml. of conc. HCl and 65 ml. of isopropanol, add water to 100 ml.

8

Milk

The first food naturally taken by the newborn baby or the young of any mammalian species is *colostrum*. This is the secretion of the mammary glands during the earliest phases of lactation. Although it may appear before parturition, it is characteristic of the second or third day *post partum* of the human mother, and is secreted in a total amount of about 100 ml. This early secretion is more viscous, richer in protein and salts, and poorer in sugar and fat than milk. The color of human colostrum is lemon yellow while that of cattle is reddish yellow. Bovine colostrum, examined just after calving, usually contains vitamin B_{12} in concentrations significantly higher than the 7 μg. per liter average found in mature milk.[1] Colostrum, unlike mature milk, is heat-coagulable, since it contains high concentrations of *globulins*, sometimes exceeding 10 per cent in cattle. Among these globulins have been identified many immune globulins.

It has been recognized for more than half a century that newborn mammals acquire immunity to certain infections by ingestion of colostrum. This mechanism appears to be more important in calves than in babies. Immune globulins are absent from the blood of newborn calves, but appear three hours after colostrum is fed. This ability to absorb significant amounts of intact protein appears in the newborn of several species, but is limited to their first 24 to 48 hours. There is evidence[2] that the absorption of immune globulins is effected by the intestinal lymphatics rather than by the portal system. The presence of *trypsin inhibitor* in colostrum[3] may aid this temporary process of passive alimentary immunization by delaying the digestive hydrolysis of protein.

In cattle, *precolostrum* may be expressed from the udder at about half-term. The globulin content is high, but the normal constituents of milk are deficient. The presence of precolostrum before lactation starts and its dilution with milk as lactation begins explain the composition of colostrum and the short duration of its production at the start of lactation. An antibody (agglutinin for *Brucella abortus*) has been fractionated from the globulins of precolostrum.[4]

Mature Milk. In human lactation, the secretion of colostrum does not continue beyond the fifth day after childbirth. There is a transitional period of about five more days, during which the components gradually change their proportions to those of mature milk. The fat content of human milk increases during the transitional period and continues to rise for the first two weeks, with no consistent variation after the third week. Increase in lactose content continues for several weeks after the establishment of mature milk flow.

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[1] Anthony, Couch, Rupel, Henderson, and Brown: *J. Dairy Sci.*, **34**, 749 (1951).

[2] Comline, Roberts, and Titchen: *Nature*, **167**, 561 (1951).

[3] Laskowski and Laskowski: *J. Biol. Chem.*, **190**, 563 (1951).

[4] McDougall: *Biochem. J.*, **44**, 531 (1949).

COMPOSITION OF MATURE MILK
Representative values in g. per 100 ml. whole milk*

Constituent	*Human*	*Cow*	*Goat*
Total solids	12.4	12.8	13.6
Protein	1.2	3.3	3.4
Lactose	7.0	4.8	4.7
Fat	3.8	3.8	4.1
Ash	0.21	0.71	0.77

* Summarized from Macy, Kelley, and Sloan, *The Composition of Milks* (*Bull. Nat. Research Council,* **#119**), Washington, D.C., 1953.

In the dairy cow, the change from colostrum to mature milk is a continuous process, taking from 5 to 12 days. The composition of mature milk is subject to considerable variation, depending upon the breed and the state of nutrition of the dairy animal, the duration of lactation, and the climate. Therefore, the accompanying table and similar tabulations purporting to represent the composition of milk must be recognized as simplified collections of averages, deviations from which are to be expected in the analysis of any particular sample of milk. The extent of some of these deviations will be indicated in following sections. For much more detailed tables, including many constituents not considered here, consult the monograph of Macy, Kelley, and Sloan, from which the figures of the table above were summarized. The customary pooling of market milk tends to minimize variations in composition. In the process of pooling, the fat content is usually standardized. Since milk is an important article of commerce, and since its nutritive value is of importance to the public health, standards of composition have been enacted into law. Legal definitions of milk vary in different jurisdictions, but many are consistent with the following:

> Milk is hereby defined to be the lacteal secretion, practically free from colostrum, obtained by the complete milking of one or more healthy cows, which contains not less than 8¼ per cent milk solids—not fat, and not less than 3¼ per cent milk fat. (*Milk Ordinance and Code—1953*, Recommendations of the Public Health Service.)

By this and most public regulations in the United States, the designation "milk" is limited to the milk of the cow, with the legal implication that the milks of other species may not be sold simply as "milk." Furthermore, in many jurisdictions, milk of other species is not subject to the requirements of quality and cleanliness imposed by law.

Milk Sugar. The only carbohydrate present in nutritionally significant amount in milk is *lactose*, a disaccharide consisting of one glucose unit and one galactose unit with the glycoside link between the carbon 4 of glucose and the carbon 1 of galactose. This glycoside link possesses the beta configuration, so that the full chemical name of lactose is D-glucopyranose-4-(β-D-galactopyranoside). Lactose is a reducing sugar and exhibits mutarotation, since carbon 1 of the glucose portion is free. Like other reducing disaccharides, lactose reduces acid Cu^{++} solutions more slowly than do monosaccharides, and hence can be distinguished from them by the use of Barfoed's reagent. Lactose forms a specific phenylosazone, yields a positive mucic acid test, and is not fermented by the ordinary commercial strains of yeast.

Lactose is formed by the mammary gland from glucose or glycogen.[5] The lactose content of milk is not notably altered by changes in the maternal diet or level of blood sugar. Lactose can frequently be detected in specimens of human urine collected *post partum* or during lactation.

Lactose is converted to lactic acid in the ordinary souring of milk, which is brought about by enzymes of *Streptococcus lactis* and numerous other microorganisms. The first step in this conversion is the hydrolysis of lactose by lactase in the surface of the bacterial cell.[6] The glucose is converted to glucose-6-phosphate by the usual steps. Galactose is phosphorylated by ATP to α-galactose-1-phosphate in the presence of galactokinase. In the presence of uridine diphosphoglucose, α-galactose-1-phosphate is converted to α-glucose-1-phosphate, which is converted to glucose-6-phosphate by steps which, together with subsequent glycolytic steps, are identical in lactic acid bacteria with those in muscle (see Chapter 10).[7]

The percentage of lactose varies significantly in the milks of different species. Human milk has a higher lactose content than that of dairy animals. The mean value in statistically adequate studies is close to 7 g. per 100 ml. human milk, with standard deviations less than 0.5 g. The mean for cattle and goats is slightly less than 5 g. lactose per 100 ml. milk, with somewhat larger variations. When babies are reared on dairy milk, it is customary to add carbohydrate in the form of lactose, sucrose, glucose, or partially hydrolyzed starch (maltose and dextrins).

Lactose is present in no other foodstuff than milk, except of course foodstuffs to which milk or lactose from milk may have been added. Compared with the other common sugars, lactose is less soluble and less sweet. The lack of sweetness is considered to be advantageous in the feeding of infants and invalids, since there is less tendency to cloy the appetite. Nutritional-balance studies[8] with a small group of children have indicated that calcium retention is greater on a diet containing 36 g. of lactose per day than on one otherwise equivalent but with no lactose.

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[5] Reithel, Horowitz, Davidson, and Kittinger: *J. Biol. Chem.*, **194**, 839 (1952).

[6] Reithel: Personal communication.

[7] Cori, C.: Personal communication.

[8] Mills, Breiter, Kempster, McKey, Pickens, and Outhouse: *J. Nutrition*, **20**, 467 (1940).

Milk Lipides. Fats occur in milk in the form of suspended globules, of a size easily observed with the low-power microscope. The size of the fat globules in a given specimen of milk is somewhat variable, the largest globules being about seven times the diameter of the smallest. Some variations in average size of fat globules occur in the different breeds of dairy cattle. It is apparent from the large size of the globules of fat in milk that such a suspension of fat in an aqueous system would not be indefinitely stable.

A large proportion of the fat of cow's milk rises spontaneously to the top of the container upon standing, and may be skimmed off as cream. The separation of cream is sharper and more rapid if the milk is first diluted. The centrifugal separator effects a prompt separation of cream without dilution. A sharp cream line is seldom observed when human or goat's milk is allowed to stand, but the cream can be separated by dilution and standing or by centrifugation. This difference in cream-rising, in part the result of larger fat globules in cow's milk, can be altered by *homogenization*, which is the mechanical reduction of the size of fat globules.

The mean values for fat in human, cow's, and goat's milk (see p. 220) are not far apart. In contrast to this consistency of average values in the three species, individuals vary widely in the fat content of milk, and among cattle and goats there are distinct differences according to breed. The milk of Holstein cattle tends to run below the mean value of 3.8 per cent, whereas good specimens of Jersey cows almost consistently give milk exceeding 5 per cent in fat content. The records for fat production, however, are, and long have been, held by Holsteins, since they make up for lower fat percentage by higher volume production of milk. Low fat values around 1 per cent and high values around 9 per cent have been occasionally recorded on authentic specimens of human, cow's, and goat's milk even when proper precautions have been taken to avoid sampling errors.

The fat content of cream varies according to the method of separation and is usually less than 35 per cent. Butter is a still more concentrated form of milk fat, containing more than 80 per cent. The Babcock test for fat in milk was introduced in 1890 as a practical method for routine use in dairies and creameries. High analytical accuracy was not claimed. In practice, the Babcock test has been found to give results only slightly (0.05 to 0.07 per cent) higher than the standard Roese-Gottlieb ether-extraction method, or its mechanized equivalent, the Mojonnier method. The modification of the Babcock test described in the experimental section of this chapter is not official, and would not be acceptable in establishing butterfat records or in court actions. In such situations the analyst should familiarize himself with the regulations governing butterfat analyses in his particular state or territory.

The triglycerides which compose milk fat contain a complex mixture of fatty acids, with oleic and palmitic acids quantitatively predominant. The fat of human milk is characterized by a somewhat higher proportion of oleic acid, and a relative deficiency of short-chain fatty acids (butyric, caproic, caprylic, and capric) as compared to goat's or cow's milk. Comparing the latter two species, cow's-milk fat has much more stearic acid, somewhat more butyric acid, and notably less caproic, caprylic, and capric acids than goat's milk. These differences in fatty acid distribution are probably of little nutritional significance, but become apparent when one compares the butyric odor of rancid cow butter with its goat counterpart. Of greater significance are certain fatty acids with more than one double bond. Rats develop dermatitis when fed an otherwise adequate diet deficient in such multiply unsaturated fatty acids. Hansen and Burr[9] list linoleic acid, linolenic acid, and arachidonic acid as normal dietary components which in very small amounts will relieve this deficiency. The milks of all three species discussed contain arachidonic acid, and the concentration in goat's and cow's milk is somewhat higher than in human milk. The presence of linoleic acid in milk has been verified.[10]

Cholesterol is present in small amounts in milk. The mean value for cow's milk is 11 mg. per 100 ml.[11] Both fatty acids and cholesterol are synthesized in the lactating mammary gland, as demonstrated by the rapid incorporation into these milk lipides of injected carboxy-C^{14} acetate, both in the intact lactating goat[12] and in the perfused isolated udder of the cow.[13]

Milk Proteins. Human milk is notably low in protein content, with a mean concentration of 1.2 g. per 100 ml. and rare variations below 1 or above 2 g. The mean values for cow's and goat's milk are 3.3 and 3.4 g. per 100 ml., most analyses falling within the limits of 2 and 5 g. In the preparation of formulas for feeding young infants, dilution of dairy milk is a customary but not universal practice. Although such dilution, together with the addition of carbohydrate, yields a mixture more closely resembling human milk, the emphasis in present-day infant feeding is upon meeting the nutritional needs of the infant rather than upon close imitation of the composition of human milk.

The chief and characteristic protein of milk is *casein*, a complex of phosphoproteins which makes up a third of the protein of human milk, three-fourths of the protein of goat's milk, and a little over five-sixths of the protein of cow's milk. Casein is definitely not a single molecular species, since three electrophoretic peaks have been distinguished (see p. 7). Fresh milk, at its pH of 6.6 ± 0.2, has as its chief colloidal component a complex of calcium caseinate and phosphate.[14] If the milk is brought by natural souring or by cautious acidification to pH 4.55, the isoelectric point of casein, this complex is broken up and casein is precipitated. The product of such a precipitation may be designated *isoelectric casein*. Casein can also be precipitated by the action of certain proteolytic enzymes, the best known of which is *rennin* (*rennet* to cheese-makers) obtained from the fourth stomach (*abomasum*) of nursing calves. Rennin does not occur in the human being, but other enzymes, in particular pepsin and chymotrypsin, carry on the same function. Crystallized rennin has been shown to be a proteolytic enzyme which, in causing milk to curdle, hydrolyzes one peptide bond per 10,000 in casein.[15] The precipitate resulting from the coagulation of milk by proteolytic enzymes is therefore not identical with isoelectric casein, but has been subjected to a mild degree of proteolysis. This product is called *paracasein*, and at pH values above the isoelectric point, is precipitated as *calcium paracaseinate*. Further proteolysis of casein yields the usual intermediate products of protein hydrolysis, except that *phosphopeptones* containing phosphoserine have been isolated from tryptic digests of casein. The caseins of milks of different species are not identical in composition.

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[9] Hansen and Burr: *J. Am. Med. Assoc.*, **132**, 855 (1946).

[10] White and Brown: *J. Am. Oil Chemists Soc.*, **26**, 385 (1949).

[11] Nataf, Mickelsen, Keys, and Petersen: *J. Nutrition*, **36**, 495 (1948).

[12] Popjak, French, and Folley: *Biochem. J.*, **48**, 411 (1951).

[13] Cowie, Duncombe, Folley, Glascock, Massart, Peeters, and Popjak: *Biochem. J.*, **49**, 610 (1951).

[14] Leviton and Haller: *J. Phys. & Colloid Chem.*, **51**, 460 (1947).

Except for some cottage cheese (Dutch cheese or schmierkäse), which is usually crude isoelectric casein, the varieties of cheese on the American market are made from the calcium paracaseinate obtained by rennin coagulation of milk. *Cheddar* is obtained by rennin coagulation of milk, preferably pasteurized while fresh and then slightly soured by a starter of lactic acid bacteria. Later processes include cutting the curd, heating to about 40° C., straining, and *cheddaring*, which consists of matting the cheese together by piling, cutting, and repiling. During the final curing process, *Lactobacillus casei* overgrows other organisms and produces the characteristic flavor. *Swiss cheese* is started in a similar manner, but is cultured with *Propionibacterium shermanii* to produce the "eyes" and the sweetness. There is no cheddaring process, but the curd is cut and cooked firm. Curing takes three to six months, during which time bacteriological errors may occasionally produce such anomalies as "blind" cheeses, "stinkers," and "blowers." Although in France *Roquefort* is often made from ewe's milk, American blue cheeses are made from cow's milk, and are started like cheddar. The cut curd is drained, placed in small perforated forms, and sprinkled with *Penicillium roqueforti*. Further ripening and proper mold-mottling requires carefully controlled curing for six to nine months. The ripening of *Limburger* depends upon *Bacterium linens* and other naturally occurring bacteria of the milk acting upon a rennin curd produced from fresh milk without a starter, and takes about two months. Yeasts growing on the cheese surface provide essential nutrients for *B. linens*.[16] *Camembert* is the product of natural bacteria working inside the cheese while the mold *Penicillium camemberti* grows on the outside.

Casein originates in the lactating mammary gland. Certain of its component amino acids have been shown by isotopic identification to be taken up from the free amino acids of the blood,[17] and not from plasma proteins. The phosphorus of casein has similarly been shown to be derived from the inorganic phosphate of the blood. In the goat, about two hours are required for the transport of these substances from circulating blood to the casein of the milk.

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[15] Nitschman and Varin: *Helv. chim. acta*, **34**, 1421 (1951).

[16] Purko, Nelson, and Wood: *J. Dairy Sci.*, **34**, 699 (1951).

[17] Barry: *J. Biol. Chem.*, **195**, 795 (1952).

Proteins other than casein constitute more than half of the proteins of human milk, and an appreciable fraction of the proteins of dairy milk. True globulins have already been mentioned in connection with colostrum. After the removal of casein from cow's milk by isoelectric precipitation, the remaining whey is found to contain 0.6 to 0.7 per cent protein, of which roughly one-fourth is globulin, as shown by its being precipitated when the whey is saturated with magnesium sulfate at pH 7; one-half is albumin, which is not so precipitated but is heat coagulable; and the remaining fourth is proteose, which is not heat-coagulable. From the "albumin" fraction has been crystallized a β-lactoglobulin which is probably identical with other preparations described as lactalbumins. The amino acid composition of β-lactoglobulin is quite different from that of any plasma protein, but the immune globulins of colostrum are similar in composition to their plasma counterparts.

Among the proteins of milk are included numerous *enzymes.* The pattern of enzymes is quite different in milks of different species. Aldolase and xanthine oxidase (Schardinger enzyme) are present in cow's milk but not in human milk. Catalase and peroxidase are present in raw milks. Human milk, particularly rich in amylase, promptly liquefies starch paste. The lipase of human or bovine milk brings about a slow liberation of free fatty acids from milk fat. Cow's milk collected in mid-lactation contains from 80 to 120 King-Armstrong units of alkaline phosphatase (phosphomonoesterase) per 100 ml., compared with a range of 0.7 to 16.2 units in human milk and a mean of 4.8 units (Kon and Mawson). The phosphatase content of cow's milk has been made the basis of tests for adequate pasteurization, since the enzyme is readily destroyed by heating.

ESSENTIAL AMINO ACIDS IN WHOLE MILK PROTEINS
(Expressed as per cent of total protein (N = 16))*

	Human Milk Proteins	*Cow's Milk Proteins*
Arginine	4.3	4.0
Histidine	2.8	2.8
Isoleucine	7.2	7.1
Leucine	9.8	10.4
Lysine	7.2	8.3
Methionine	2.2	3.0
Phenylalanine	5.6	5.2
Threonine	4.6	4.5
Tryptophan	1.9	1.4
Valine	8.8	6.7

* These representative data are derived largely from Block and Bolling, *The Amino Acid Composition of Proteins and Foods*, Charles C Thomas, Springfield, Ill., 1951. The N = 16 convention is used in dietary calculations, although for milk proteins (largely casein) N = 15.7 (i.e. protein = N × 6.38) is more accurate. For additional data on individual milk proteins see table on p. 122.

The *biological value* of a protein food is defined as the ratio (expressed as percentage) of the food nitrogen retained to that absorbed (see p. 1047). It is usually determined by feeding experiments on growing rats. A high value indicates adequacy in content and availability of so-called essential amino acids. The biological values of human and cow's milk are identical within limits of experimental error, the numerical range being 85–90. This high biological value is confirmed by direct analysis of milk proteins for essential amino acids. The *essential amino acid index* of cow's-milk protein based on the relationship between its essential amino acid content and that of whole-egg protein (assumed to equal 100) is 90; the corresponding index for human-milk protein is 93. These index values are closely correlated with the biological values.[18] The table on p. 225 demonstrates the adequacy of whole milk in those amino acids generally regarded as indispensable in the human diet. This question is more fully discussed in Chapter 33.

Inorganic Milk Components. The crude figures for ash in the table on p. 220 indicate that the concentration of total minerals is more than three times as great in the milk of dairy animals as in human milk. The important bone-mineral elements, calcium and phosphorus, are both present in milk in nutritionally significant amounts and in a ratio suitable for effective absorption and utilization.

Calcium is present in human milk in concentrations which vary through a range of 10 mg. per 100 ml. on either side of a mean of 30 mg. Cow's or goat's milk averages close to 130 mg. per 100 ml. The calcium of milk is present in part as calcium ion, and in part bound as a colloidal complex with protein and phosphate.

Phosphorus has a mean value of 13 mg. per 100 ml. in human milk, with a standard deviation of 1.9 mg. (Kon and Mawson), but is found in amounts averaging 100 mg. per 100 ml. of dairy milk, 70 to 80 per cent of the total phosphorus being in the form of inorganic phosphate. The remainder includes phosphoprotein, phospholipide, and ester phosphorus. A variable portion of the inorganic phosphate is separable by ultrafiltration as a part of the colloidal calcium caseinate-phosphate complex.

During that period of early life when milk is the only food taken, *potassium* is equal in importance to the bone minerals. At all ages, the body contains more potassium than sodium, but the difference is greater with growth. Human milk contains approximately 11 milliequivalents of potassium and 5 milliequivalents of sodium per liter; this ratio seems to be optimal for the growth of the infant. Dairy milk contains these ions in comparable ratio, but in concentrations about three times higher. *Chloride* ion is present in highly variable amounts. *Magnesium* averages 4 mg. per 100 ml. of human milk, and three to four times this concentration in dairy milk. The *sulfur* of milk is almost entirely in the form of the sulfur-containing amino acids of milk proteins, and averages 30 mg. per 100 ml. of cow's milk, and about half this value in human or goat's milk.

In addition to those mentioned above, a number of other elements are present in small and variable amounts. Milk is notoriously deficient in *iron*. Analytical figures from different laboratories are widely divergent, but for cow's milk, a concentration of 0.32 mg. per kg. is representative.[19] The iron content of milks of other species is comparable. The full-term human infant born of an adequately nourished mother has a store of iron for about six months of growth on a milk diet without developing an iron-deficiency anemia. After six months, supplementary iron-containing foods should be introduced into the diet. *Copper* occurs in raw cow's milk in about the same concentration as iron. Higher concentrations may result from contact with copper or brass during pasteurization or other processing. Copper in excess of 0.5 mg. per kg. produces a tallowy flavor and loss of ascorbic acid in milk. Average values for other trace elements in cow's milk are given by Archibald[20] as follows:

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[18] Oser: *J. Am. Dietet. Assoc.*, **27**, 396 (1951).

[19] Johnston: *Food Res.*, **9**, 212 (1944); Johnston, Gellman, and Strom: *J. Biol. Chem.*, **175**, 343 (1948).

Manganese	22	μg. per liter
Zinc	3900	" " "
Cobalt	0.6	" " "
Molybdenum	73	" " "
Nickel	none	

The presence of iodide has been demonstrated in the milk of women and of animals. Electrophoretic evidence has indicated that milk contains an iodinated protein.[20a]

Vitamins in Milk. Milk and milk products are outstanding sources of certain vitamins, and less reliable for the supply of others.

Thiamine is present in human milk in amounts which roughly reflect the vitamin B_1 intake of the mother. At moderate levels of intake, about 10 per cent of ingested thiamine is secreted into the milk (Kon and Mawson). The concentration of total thiamine (in the milk of mothers whose intake is between 1.5 and 2 mg.) usually lies between 14 and 19 μg. per 100 ml. In early lactation, from 50 to 90 per cent of the thiamine of human milk is in a combined form which is not reactive in the thiochrome test, but becomes reactive after enzymic hydrolysis with *takadiastase*, which contains numerous enzymes including phosphatases and proteases. In general, high content of combined thiamine is associated with low values of milk phosphatase. Both free and combined thiamine are measurable by bioassay. The proportion of free thiamine increases with the duration of human lactation.

The milk of goats and cattle contains somewhat more than twice the concentration of thiamine in human milk. About half is in the form of free thiamine in cow's milk, and about one sixth in goat's milk. Thiamine pyrophosphate added to cow's milk is promptly hydrolyzed in the presence of the enzymes pyrophosphatase and phosphatase. A portion of the combined thiamine in cow's milk is bound to protein and liberated by hydrolysis with pepsin. In the cow, total thiamine of the milk is higher in early lactation than in the later months. As in human milk, the thiamine of cow's milk varies with the amount supplied in the diet, but the cow has the additional possibility of supplementation by thiamine formed by microbiological synthesis in the rumen. One quart of milk contains about one-fourth the daily human adult requirement of thiamine.

Riboflavin is the one vitamin for which dairy milk and milk products are the richest everyday source, exceeded in riboflavin concentration only by liver and yeast. The source of this abundance of riboflavin is bacterial action in the rumen. In cattle, a day's output of riboflavin in the milk has been found to be up to ten times the dietary intake. Most samples of dairy milk contain between 100 and 200 μg. of riboflavin per 100 ml. Cheese and other milk products, including evaporated milk, are excellent sources of riboflavin. If milk and milk products are excluded from the usual American diet, the riboflavin requirement is not met. The riboflavin of milk and milk products is as completely available to the human organism as is riboflavin taken in simple water solution. This is not true of the riboflavin in some other common foodstuffs.[21]

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[20] Archibald: *J. Dairy Sci.*, **34**, 1026 (1951).

[20a] Middlesworth, Tuttle, and Threlkeld: *Science*, **118**, 749 (1953).

The riboflavin content of human milk is much lower than that of cow's milk, usually between 20 and 30 μg. per 100 ml. if no supplementary riboflavin is ingested. Since the contribution of riboflavin to the human organism from bacterial synthesis in the large intestine is minimal, the riboflavin content of human milk is determined by the riboflavin of the diet.

Nicotinic acid is present in cow's milk in a concentration usually slightly less than 100 μg. per 100 ml. Somewhat higher concentrations are reported for goat and human milk. It is obvious that the recommended daily adult intake of 10 to 15 mg. could not be met with milk alone, and one might wonder how the nursing young avoid deficiency. A part of the answer is that many animals, including man, can meet part of the nicotinic acid requirement by synthesizing it from tryptophan. Pellagra-producing diets have been characterized by low content of tryptophan as well as of nicotinic acid. Milk, although low in nicotinic acid, is high in tryptophan (40 to 50 mg. per 100 ml. in cow's milk).

Cattle appear to be able to meet their entire requirement for *pantothenic acid* by bacterial synthesis in the rumen. Dairy milk contains 300 to 400 μg. of pantothenic acid per 100 ml., human milk about half this amount. The *pyridoxal* group of vitamins (vitamin B_6) is present in milk in variable amount, most reports giving mean values between 50 and 70 μg. per 100 ml. for cow's milk. *Biotin* is present in milk in quite variable concentrations up to 10 μg. per 100 ml. The mean *folic acid* and *vitamin B_{12}* contents[22] for human milk have been reported as 0.71 and 0.41 μg. per liter; for cow's milk, 1.3 and 6.6 μg. per liter, and for goat's milk 2.7 and 0.12 μg. per liter. All the B vitamins mentioned in this paragraph are those of which an uncomplicated dietary deficiency has not been observed in man, and which in large part may be supplied to man by bacterial synthesis in the intestine; hence the values given are of more academic than nutritional significance.

The *ascorbic acid* content of cow's and goat's milk, when fresh, is about 2 mg. per 100 ml., which decreases to less than 1 mg. by the time the milk is made available through usual retail outlets.[23] For infants, and for adults living on milk diets, supplementation with ascorbic acid is necessary for optimal intakes. Human milk, depending somewhat upon the nutrition of the mother, usually runs higher in ascorbic acid than the milk of dairy animals. On intakes of vitamin C ranging from 43 to 106 mg.

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[21] Everson, Pearson, and Matteson: *J. Nutrition*, **46**, 45 (1952).

[22] Collins, Harper, Schreiber, and Elvehjem: *J. Nutrition*, **43**, 313 (1951).

[23] Holmes: *J. Am. Dietetic Assoc.*, **27**, 578 (1951).

daily, 16 mothers were found to put out from 19 to 46 mg. of ascorbic acid in the milk per day, at concentrations ranging from 1.9 to 5.8 mg. per 100 ml. (Kon and Mawson).

Vitamin A, being fat-soluble in contrast to the vitamins previously mentioned, is found in association with milk fat. Human milk may contain from 50 to somewhat over 200 units of vitamin A per 100 ml., the concentration of the vitamin being correlated with the fat content of the milk. The *carotenoids* which are responsible for the yellow pigmentation of milk fat are to a varying degree *provitamins A*. Human milk contains from 7 to 25 μg. of carotenoids per 100 ml. The milk of cattle contains vitamin A and provitamins A in concentrations which, though comparable to those in human milk, are highly variable, depending upon the composition of the feed.

Vitamin D, under optimal conditions, may be present in human milk, dairy milk, or milk fat in amounts adequate to prevent rickets in infants and children. Since this is often not the case, supplementation with vitamin D is standard practice in those parts of the world where exposure of infants, mothers, or dairy animals to sunlight is inadequate. Milk is often enriched with vitamin D to provide 400 units per quart.

Vitamins K and *vitamins E* are present in measurable amounts in human and dairy milk. The concentrations of these substances in milk are not significant in human nutrition.

MILK AND HUMAN NUTRITION

The suitability of milk and milk products for human food is a logical consequence of their chemical composition. The history and experience of mankind give further evidence of the value of dairy animals to our well-being. The cow produces a protein food of high biological value and produces it with an extraordinary economy which results from her ability to utilize a high proportion of rough feed in her diet. As an extreme case, she can survive and produce milk on forage alone, although this is not the way to maintain a high milk production. Protein foods are, of course, necessary for man at all ages, but their lack is most disastrous during early childhood shortly after weaning. *Kwashiorkor*, or malignant malnutrition, occurs in children in those areas where the proteins of high biological value supplied by such foods as milk, meat, and egg are lacking, and where starchy foods are the dietary staples.[24] This disease is characterized by extreme fatty degeneration of the liver, with permanent scarring (cirrhosis) in those who survive. Although kwashiorkor is a disease of multiple dietary deficiencies, usually with adequate caloric intake, it responds poorly to vitamin therapy, and is best treated or prevented by protein foods.

Milk has a justified reputation as a "protective" food, meaning that it protects the individual from several possible dietary deficiencies, including those of protein, calcium, and riboflavin. Like many other good things, milk can be used unwisely. Older children require more protein than is supplied by a quart of milk, and it is wise not to depend upon milk alone as a protein source, but to supplement it with more concentrated protein foods. It should also be recalled that milk is not a dependable source of iron, thiamine, ascorbic acid, or vitamin D (except vitamin D milk) in the diet of growing children. Iron-deficiency anemia is quite characteristic of infants who have been kept too long on unsupplemented breast or bottle feeding.

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[24] Meiklejohn and Passmore: *Ann. Rev. Med.*, **2**, 129 (1951).

The use of *human milk* has obvious advantages in the feeding of human infants, including optimal adaptation to nutritional needs and almost complete freedom from bacterial contamination. In lactation, the first week is critical for the mother, who should avoid overeating or other possible causes of digestive disturbances. Later, the diet should be ample and nutritious, but there need be no excessive gain in the mother's weight. The addition of one quart of dairy milk to her usual diet is a common and usually satisfactory recommendation. The average daily production of milk by the human mother ranges from around 300 ml. in the first week to 900 ml. in the twentieth week. The variation from these mean figures is extreme, and outputs of more than a gallon a day have been reported.

Cow's milk is the basis of most prepared foods for infants. The reduction in the mortality of bottle-fed babies has been one of the untrumpeted triumphs of preventive medicine. As recently as 1922, the mortality of bottle-fed babies was four times that of breast-fed babies. At present there is no significant difference. Pasteurization (heating to 143° F. for 30 minutes) destroys pathogenic bacteria without coagulating the whey proteins. This is an adequate method, when combined with proper handling after pasteurization, of rendering the milk suitable as a food for adults. It eliminates the possibility of transmission of brucellosis or bovine tuberculosis to human beings. It is not an adequate treatment of milk for young babies, for whom all milk, whether raw or pasteurized, is customarily boiled. This reduces the toughness of the casein curd (see p. 366) and kills all bacteria. There is no nutritional disadvantage to the boiling of milk other than the complete destruction of ascorbic acid, which is customarily given in a supplementary food.

Goat's milk is used as a food to a limited extent in the United States. A considerable amount is produced in small dairies, chiefly for home use. When goat's milk is available on the market, the price is usually higher than that of cow's milk. The nutritive value of goat's milk is so close to that of cow's milk that it is impossible to make a choice on this basis alone. Goat's milk has often been used successfully in the feeding of infants or children who show allergy to cow's milk. Many other therapeutic claims have been made for goat's milk, but acceptable evidence for them is lacking. The claim of easier digestibility compared to cow's milk has some basis in fact, since the fat particles of goat's milk are smaller, and the curd tension (toughness of precipitated casein) is less as compared with unhomogenized cow's milk. One often-mentioned disadvantage of goat's milk is apparently fictitious—so-called "goat's milk anemia." In experiments with both rats and babies, no difference has been found in the outcome of feeding with cow's and goat's milk. Either is nutritionally satisfactory, and both require supplementation with iron and with vitamins C and D. Under the very best stabling conditions for both species, as for example for the experimental herds at Beltsville, it is possible to keep goats cleaner than cows; hence the bacterial counts of goat's milk are lower under such test conditions. Under ordinary stabling conditions, goat's milk has no less opportunity to be contaminated than cow's milk. Goats are not susceptible to bovine tuberculosis, but otherwise any milk-borne disease can be carried as effectively by goat's milk as by cow's milk; hence pasteurization or boiling is as necessary for the milk of one species as for that of the other. Inspection of cow's milk and of the dairies where it is produced is almost universal in American communities, but goat's milk is often not included in the regulations governing such inspections.

Evaporated milk is prepared by concentration under reduced pressure to slightly less than half the original volume. The concentration of dissolved solids is therefore slightly more than twice that of the original milk. For purposes of infant feeding, evaporated milk may be used in half the amount specified for whole milk, reconstituted to the specified volume with boiled water. Most brands of evaporated milk sold at retail are fortified to provide 400 U. S. P. units of vitamin D_1 per reconstituted quart, and this is indicated on the label. Evaporated milk is sterilized at high temperatures after being sealed in cans, and under present-day conditions of manufacture is rarely subject to spoilage from incomplete sterilization. Even though the milk is sterile, undesirable alterations of viscosity may occur on storage, sometimes with separation of proteins into an insoluble gel. These changes are unlikely if evaporated milk is used within two years, and if it is stored at temperatures below 60° F.[25]

Canned fresh whole milk, which will keep four to six months without refrigeration, is sterilized by bringing its temperature almost to 300° F. for a few seconds in a specially designed heat-exchanger. The canning is done under sterile conditions. This type of canned milk has no cooked flavor. At the time of this writing, this product is not sold extensively at retail in the United States, but is processed chiefly for the armed services.

The original *condensed milk* (1858) was concentrated by evaporation under reduced pressure and preserved by the addition of sucrose. It was not originally sold in cans, but in bulk after the manner of sale of milk at that time. For many years this product has been available at retail, canned and heat-sterilized. The concentration of milk components in present-day condensed milk is a little higher than in evaporated milk, and the percentage of added sucrose is about 40 per cent.

The better grades of *dried milk* are prepared by very rapid drying under reduced pressure, which preserves the vitamin potency as well as other nutrients. Such products are satisfactory for human food and for infants' formulas, when produced under proper sanitary conditions. Cheaper grades, intended for animal food, are produced under less strict standards of quality and sanitation.

Storage of fluid milk or of milk concentrates at very low temperatures (−5° to −15° F.) is a satisfactory method of preservation which does not produce undesirable changes in flavor or physical properties.[26] The use of cathode rays has been found effective in sterilizing milk, and is claimed to be without effect upon flavor.[27]

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[25] Webb, Deysher, and Potter: *J. Dairy Sci.*, **34**, 1111 (1951).

[26] Tracy, Hetrick, and Krienke: *J. Dairy Sci.*, **33**, 832 (1950).

State laws in the United States require that *ice cream* contain a specified percentage of milk fat, the percentage varying from 8 to 14 in different jurisdictions. Frozen preparations with lower fat content are sold as ice milk or sherbet.

Determining the freezing point of milk is a simple physical test which reveals dilution with water. Observation in a mixed milk specimen of a freezing point higher than the limiting value of −0.520° C. indicates that such dilution has been made. If the sample is from a single cow, there is a remote chance that this limiting value may be exceeded without the addition of water. If the sample is a mixture of the milk of six or more cows, a freezing point above the limiting value is definite evidence of watering, and if the sample is from forty cows or more, a freezing point above −0.540° C. raises strong suspicion of watering.[28]

Economics. The milk-producing cows of the United States number close to 25,000,000 and their output is estimated at over 14,000,000,000 gallons per year, which brings to American dairymen an annual gross income of more than $3,760,000,000. Estimates released by the Milk Industry Foundation apportion the 1950 milk output as having been used as follows:

Fluid milk and cream	
sold in cities and villages	37.3%
used on farms where produced	10.1%
Butter	
creamery-made	22.7%
farm-made	4.3%
Cheese	9.5%
Ice cream	5.1%
Evaporated, condensed, and dried milk	6.4%
Milk fed to calves on farms	2.7%
Milk for other uses	1.9%

The figures above do not tell the whole story, since skim milk, buttermilk, and whey remain after cream, butter, and cheese have been made. Lactose for use in the manufacture of food and medicinal products is separated from such residues and marketed at a rate of 40,000,000 pounds a year in the United States. About half this amount of casein is similarly obtained, and is used for human and animal food, and in pharmaceutical, paper, paint, and adhesive products. Hydrolyzates of casein are used in special diets, in food flavoring, and as nutrients in industrial microbiology. The whey proteins are also separated, and are used in food products and for many industrial purposes.

EXPERIMENTS ON MILK

1. Phosphatase Test for Pasteurization** (**Cornell Method**[29]): **Principle. The destructive effect of heat on the natural phosphatase in raw milk is used as the basis for testing the efficiency of pasteurization. Phosphatase activity is measured by the hydrolysis of disodium phenylphosphate and colorimetric estimation of the released phenol. Dairy product samples are incubated with the substrate buffered at pH 9.5 to 9.7, and then the protein is precipitated. After filtration, the filtrate is brought back to an alkaline reaction with carbonate, and BQC[30] is added. Blue color is produced if sufficient phosphatase was present in the milk to split the disodium phenylphosphate substrate during incubation. The procedure as given here applies to milk. With minor modifications (given in the original paper) the Cornell test can be applied to other dairy products.

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[27] *Chem. Eng. News*, **29**, 2818 (1951).

[28] Lythgoe: *J. Assoc. Offic. Agr. Chemists*, **55**, 442 (1952).

[29] Kosikowsky: *J. Dairy Sci.*, **34**, 1151 (1951). Reproduced by permission of the author and the *Journal of Dairy Science*.

Procedure.[31] a. SAMPLING AND INCUBATING (LONG METHOD). **For milk and other fluid dairy products, 1 ml. of milk or milk product is transferred to a 25 × 150 mm. test tube. Add 10 ml. of warm (40° C.) carbonate buffer substrate and 4 drops of U. S. P. chloroform. A piece of parchment paper is fitted over the tube with the aid of a rubber band, and the milk is incubated at 32° to 37° C. for 18 to 24 hours.**

b. PRECIPITATION. **After incubation, 1 ml. of trichloroacetic—HCl precipitant is slowly added to the tube. The resulting protein precipitate is filtered off through Whatman No. 42 paper (11 cm.).**

c. COLOR DEVELOPMENT. **Five ml. of the clear filtrate is pipetted into a 16 × 150 mm. test tube. One ml. of $CuSO_4$–"Calgon" solution and 5 ml. of 8 per cent Na_2CO_3 are added. Then 2 drops of BQC solution are placed in this solution. The tubes after mixing are inserted in a water bath at 37° C. for 15 minutes. Color development is measured after this interval against suitable color standards or in a colorimeter.**

INTERPRETATION OF RESULTS. All final color readings are, after consideration of control values, multiplied by a factor of 1.2 to convert to μg. phenol per 0.5 ml. Any value over 5μg. per 0.5 ml. milk or other fluid dairy product for the long procedure, or 2 μg. per 0.5 ml. for the short method, is tentatively considered to indicate either underpasteurization or the presence of raw-milk products, or a combination thereof. The short method, even to the extent of using samples of the same size, is conducted in the same way as the long method except that (a) incubation is carried out for 1 hour at 37° to 38° C., and (b) chloroform is omitted.

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[30] See 2,6-Dibromoquinonechloroimine solution (BQC) under footnote 31.

[31] *Reagents and Materials:*

Carbonate Buffer Substrate: Dissolve 11.50 g. anhydrous Na_2CO_3, 10.15 g. anhydrous $NaHCO_3$, and 1.09 g. pure disodium phenylphosphate in water and make up to 1 liter ($pH = 9.80$).

Trichloroacetic—hydrochloric acid precipitant: Dissolve 25 g. trichloroacetic acid crystals in water, make up to 50 ml. with water; add 50 ml. conc. HCl (approx. 36 per cent) and mix thoroughly.

Sodium carbonate solution (8 per cent): Dissolve 80 g. anhydrous Na_2CO_3 in water and make up to 1 liter.

Copper sulfate-"Calgon" solution: Dissolve 500 mg. $CuSO_4 \cdot 5H_2O$ and 20 g. "Calgon" (sodium hexametaphosphate) crystals (tech.) in water and make up to 1 liter.

2,6-Dibromoquinonechloroimine solution (BQC): Dissolve 50 mg. BQC in 10 ml. absolute ethyl or methyl alcohol and store in dark bottles.

Color standards: Make the following solutions as preliminary to making standards—

a. Stock phenol solution: Dissolve 1 g. phenol crystals in water and make up to 1 liter.

b. Buffer solution: Make 1 liter of carbonate buffer containing 11.50 g. Na_2CO_3 and 10.15 g. $NaHCO_3$.

c. Diluted phenol solution: Using 4 ml. of stock phenol solution (a), make up to 500 ml. with buffer solution (b). This solution contains 8 μg. phenol per ml.

Preparation of color standards: Place in clean 16 × 150 mm. test tubes 0.5 to 5 ml. portions of Diluted Phenol Solution (c). Add enough Buffer (b) so that total volume of liquid in each tube is 10 ml. Then add 1 ml. of copper sulfate–"Calgon" solution to each tube. Finally, add 2 drops of BQC and 4 drops U. S. P. chloroform and mix. Let stand for 15 minutes at 37° C. Seal tubes with paraffin wax and store in refrigerator. Tubes containing 0.5, 1.0, 1.5, 2.5, and 5.0 ml. portions of diluted phenol solution will produce standards of 4.0, 8.0, 12.0, 20.0, and 40.0 μg., respectively, after final color development. Color standards of 2.0 and 5.0 μg. can be easily obtained by making 4.0 and 20.0 μg. solutions without color development and diluting these with sufficient Buffer Solution (b). Color is then developed in 10-ml. portions by adding copper sulfate–"Calgon" solution and BQC.

To obtain alcohol color standards, 5 ml. of butyl alcohol are added to a duplicate series of final aqueous color standards. The tubes are inverted ten times to extract color, and the cork stoppers are sealed with wax.

CONTROLS FOR LONG AND SHORT METHODS. One ml. of milk, preferably from pasteurized stock, is placed in a tube, heated to 78° C. (170° F). for 15 seconds in a water bath and then cooled immediately. This heated control is tested by the same method as employed for samples of unknown history.

ALCOHOL EXTRACTION. If necessary, butyl alcohol extraction may be used in either the long or short method, especially on critical values. Five ml. of *n*-butyl alcohol is added to the test tube containing the aqueous colored solutions, and the latter is inverted ten times. The clear layer appears without centrifuging and is compared against alcohol standards. Alcohol extraction is preferred when a colorimeter is not used, as aqueous phenol standards for all methods deteriorate, as a rule, relatively rapidly.

2. *Influence of Gastric Rennin on Milk.* Prepare a series of five tubes as follows:

a. 5 ml. of fresh milk + 0.2 per cent HCl (add drop by drop until a precipitate forms).

b. 5 ml. of fresh milk + 5 drops of rennin solution.[32]

c. 5 ml. of fresh milk + 10 drops of 0.5 per cent Na_2CO_3.

d. 5 ml. of fresh milk + 10 drops of ammonium oxalate solution.

e. 5 ml. of fresh milk + 5 drops of 0.2 per cent HCl.

To each of the tubes (c), (d), and (e) add 5 drops of rennin solution. Place the whole series of five tubes at 40° C. and after 10 to 15 minutes note what is occurring in the different tubes. Give a reason for each particular result.

3. *Preparation of Casein.*[33] Into a 600-ml. beaker introduce 200 ml. of skimmed (or centrifuged) milk. Add an equal volume of water. Add from a pipet very carefully, drop by drop with thorough stirring, 10 per cent HCl until a flocculent precipitate forms. (Casein precipitates best at a point slightly more acid than its isoelectric point of pH 4.55.) From 3 to 5 ml. of acid are commonly required. In milk, casein functions as an acid and exists as K and Ca caseinates, from which compounds it is released by the acid. As the isoelectric point is passed, however, the casein begins to function as a base and go into solution as casein hydrochloride. Hence excess of acid must be avoided. If too much acid be added, run in, drop by drop, 10 per cent NaOH solution until precipitation occurs and a clear supernatant fluid is obtained. Allow the precipitate to settle, decant the supernatant fluid, and reserve it for use in later (4–6) experiments. Filter off the precipitate of casein and remove the excess of moisture by pressing it between filter papers. Transfer the casein to a small beaker, add enough 95 per cent alcohol to cover it, and stir for a few moments. Filter, and press the precipitate between filter papers to remove the alcohol. Repeat the extraction with alcohol, making sure that the casein is in a finely divided condition. Transfer the casein again to a small dry beaker, cover the precipitate with ether and heat on a water bath, with any flames turned out, for ten minutes, stirring continuously. Filter (reserve the filtrate), and press the precipitate as dry as possible between filter papers. Open the papers and allow the ether to evaporate spontaneously. Grind the precipitate to a powder in a mortar. Upon the casein prepared in this way make the following tests:

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[32] Any commercial rennin or rennet preparation or an extract of the gastric mucosa of the pig may be employed.

[33] For the preparation of very pure casein it is better if the milk be centrifuged several times in a separator, then a small amount of dilute alkali added, and the milk centrifuged several times more. Casein is also purified by repeated solution in dilute alkali and reprecipitation by acid.

a. SOLUBILITY. Try the solubility in water, sodium chloride, dilute acid, and alkali.

b. MILLON'S REACTION. Make the test according to the directions given on p. 169.

c. CYSTINE AND CYSTEINE SULFUR. Test for cystine and cysteine sulfur according to the directions given on p. 168.

d. FUSION TEST FOR PHOSPHORUS. Test for phosphorus by fusion according to the directions given on p. 212.

4. *Coagulable Proteins of Milk.* Place the filtrate from the original casein precipitate in a casserole and heat on a wire gauze over a free flame. As the solution concentrates, a coagulum consisting of lactalbumin and lactoglobulin will form. Continue to concentrate the solution until the volume is about one-half that of the original solution. Filter off the coagulable proteins (reserve the filtrate) and test them as follows:

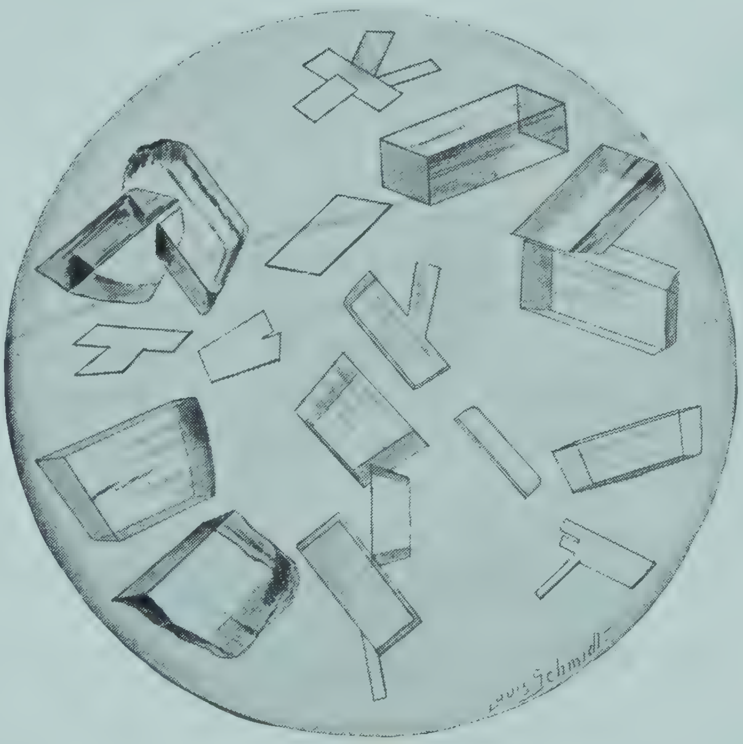

FIG. 63. LACTOSE.

a. MILLON'S REACTION. Make the test according to the directions given on p. 169.

b. CYSTINE AND CYSTEINE SULFUR. Make the test according to the directions given on p. 168. Do the coagulable proteins differ from casein in their reaction to this test? Why?

5. *Detection of Calcium Phosphate.* Evaporate the filtrate from the coagulable proteins, on a water bath, until crystals begin to form. It may be necessary to concentrate to 15 ml. before any crystallization will be observed. Cool the solution, filter off the crystals (reserve the filtrate), and test them as follows:

a. MICROSCOPICAL EXAMINATION. Examine the crystals and compare them with those in Fig. 64.

b. Dissolve the crystals in nitric acid. Test part of the acid solution for phosphates. Render the remainder of the solution slightly alkaline with ammonia, then acidify with acetic acid and add ammonium oxalate. Examine the crystals under the microscope and compare them with those in Fig. 230, p. 855.

6. ***Detection of Lactose.*** Concentrate the filtrate from the calcium phosphate until it is of a syruplike consistency, and pour it into several volumes of acetone to precipitate the lactose in crystalline form.

a. Microscopical Examination. Examine the crystals and compare them with those in Fig. 63.

b. Benedict's Test. Try Benedict's test upon the mother liquor.

c. Phenylhydrazine Test. Apply the phenylhydrazine test to some of the mother liquor according to the directions given on p. 63.

7. ***Milk Fat.*** Evaporate the ether filtrate from the casein (Exp. 3) and observe the fatty residue. The milk fat was carried down with the precipitate of casein and was removed when the latter was treated with ether. If centrifuged milk is used in the preparation of the casein the amount of fat in the ether filtrate may be very small.

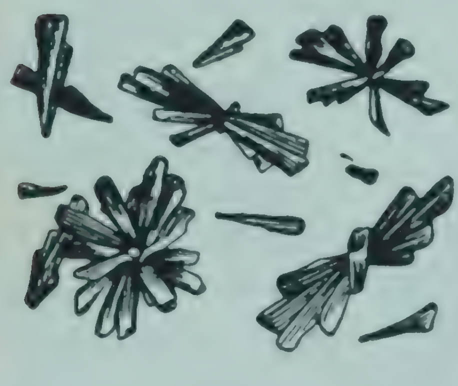

Fig. 64. Calcium Phosphate.

8. ***Saponification of Butter.*** Dissolve a small amount of butter in alcohol made strongly alkaline with potassium hydroxide. Place the alcoholic-potash solution in a casserole, add about 100 ml. of water and boil for 10 to 15 minutes or until the odor of alcohol cannot be detected. Place the casserole in a hood and neutralize the solution with sulfuric acid. Note the odor of volatile fatty acids, particularly butyric acid. Under certain conditions the odor of ethyl butyrate may also be detected.

QUANTITATIVE ANALYSIS OF MILK[34]

1. ***Collection of Human Milk for Analysis.*** There are two methods of obtaining samples of breast milk for analysis.

First Method. Express all the milk from one breast and mix thoroughly.

Second Method. Draw one ounce of milk before nursing and one ounce after nursing. Mix the two samples thoroughly. The best time for obtaining samples is 9 to 10 A.M.

2. ***Specific Gravity.*** This may be determined accurately by means of a pyknometer or more conveniently by means of a Soxhlet, Veith, or Quevenne lactometer. A lactometer reading of 32° denotes a specific gravity of 1.032. The determination should be made at about 60° F. (15.6° C.) and the lactometer reading corrected by adding or subtracting 0.1° for every degree F. above or below that temperature.

3. ***Fat.***

a. Babcock's Centrifugal Method: Principle. This method involves the breaking of the emulsion of fat in milk by means of concentrated sulfuric acid, centrifugation of the acid solution in the special tube shown in Fig. 65, and the subsequent reading of the percentage of fat in the graduated neck. Larger Babcock bottles are commonly used for testing cow's milk or cream; these are supplied with pipets calibrated to deliver 18- or 9-g. charges, respectively. The method is accurate to within 0.5 per cent.

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[34] *Official and Tentative Methods of Analysis*, 7th ed., Washington, D. C., Assoc. of Official Agricultural Chemists, 1950.

Procedure. **By means of a special narrow pipet, introduce milk into the tube up to the 5-ml. mark. Now add sufficient sulfuric acid (sp. gr. 1.83–1.834) to fill the body of the tube and rotate the tube to secure a homogeneous acid-milk solution. Fill the neck of the tube with an acid-alcohol mixture.[35] Centrifuge the tube and contents for one to two minutes and read off the percentage of fat by means of the graduated neck of the tube. If the top of the fat column is not at zero it may be brought there by the addition of hot water and a moment's recentrifugation.**

In case very rich milk (over 5 per cent fat) is under examination, it may be diluted with an equal volume of water before examination and the fat percentage multiplied by 2. In the examination of cream it is customary to dilute the sample with four volumes of water and multiply the resultant fat value by 5.

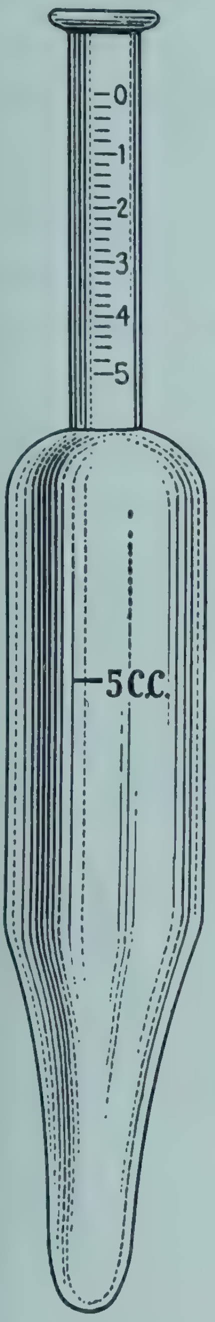

FIG. 65. BABCOCK TUBE.

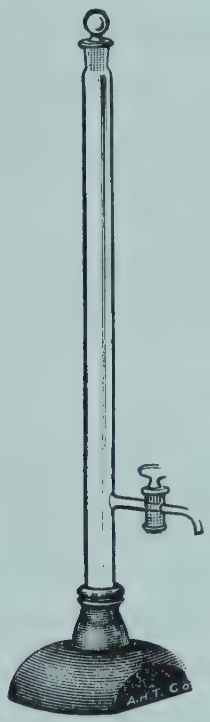

FIG. 66. RÖHRIG TUBE FOR LIQUID-LIQUID EXTRACTIONS.

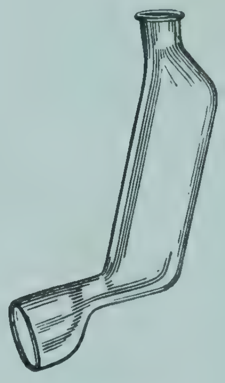

FIG. 67. MOJONNIER FLASK. Designed to permit weighing, extraction, and decantation without transfer of sample.

b. ROESE-GOTTLIEB METHOD: PRINCIPLE. The milk is made alkaline and extracted repeatedly with petroleum benzin and the filtered extract evaporated to dryness in a tared flask. This method, together with the Babcock procedure, is "official" in the A.O.A.C. Book of Methods (see footnote 34, p. 236) and is adaptable to butter, ice cream, dried milk, etc.

Procedure. **Transfer 10 g. of the sample to a Röhrig tube (Fig. 66) or a similar apparatus (Mojonnier flasks[36] (Fig. 67) are widely used in dairy laboratories), add 1.25 ml. of NH_4OH (2 ml. if the sample is sour), and mix thoroughly. Add 10 ml. of 95 per cent alcohol and mix well. Add 25 ml. of**

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[35] This mixture consists of equal volumes of amyl alcohol and concentrated hydrochloric acid.

[36] Obtained from Mojonnier Bros. Co., 4601 West Ohio St., Chicago 44, Ill.

ether, shake vigorously for 30 seconds, add 25 ml. of petroleum benzin (redistilled slowly at a temperature below 65° C.), and shake again for 30 seconds. Let stand 20 minutes, or until the upper liquid is practically clear. Draw off as much as possible of the ether-fat solution (usually 0.5 to 0.8 ml. will be left) into a flask through a small, quick-acting filter. Again extract the liquid remaining in the tube, this time with 15 ml. of each solvent; shake vigorously 30 seconds after each addition and allow to settle. Draw off the clear solution through the small filter into the same flask as before and wash the tip of the spigot, the funnel, and the filter with a few ml. of a mixture of the two solvents, in equal parts, free from suspended H_2O. To insure complete removal of the fat, a third extraction is necessary. (This third extraction yields less than 1 mg. of fat if the previous solutions have been drawn off closely.) Add a glass bead and evaporate the ethers slowly on a warm surface; then dry the fat in a boiling water oven to constant weight. Weigh the flask with a similar flask as a counterpoise. Do not wipe the flask immediately before weighing. Remove the fat completely with petroleum benzin. Deduct the weight of the dried flask with residue and bead to obtain the weight of fat. Finally, correct this weight by a blank determination on the reagents used.

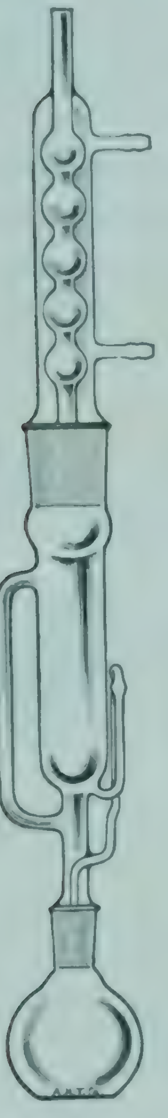

FIG. 68. SOXHLET EXTRACTION APPARATUS.

c. SOXHLET METHOD. This classical procedure is suitable for the determination of fat in solid materials such as dried milk. (Fig. 68.)

4. *Total Solids*. Introduce 2 to 5 g. of milk into a weighed, flat-bottomed platinum dish[37] (which may, if desired, contain 15 to 20 g. of pure, dry sand spread over the bottom) and quickly ascertain the weight to milligrams. Expel the major portion of the water by heating the open dish on a water bath and continue the heating in an air bath or water oven at 97° to 100° C. until the weight is constant. (If platinum dishes are employed this residue may be used in the determination of ash according to the method described below.)

CALCULATION.[38] Divide the weight of the residue by the weight of milk used. The quotient multiplied by 100 gives the percentage of solids contained in the milk examined.

5. *Ash*. Heat the dry solids from 2 to 5 g. of milk, obtained according to the method just given, over a very low flame[39] until a white or light-gray ash is obtained. If the determination is made directly on fluid milk, weigh quickly about 20 ml., add 6 ml. concentrated HNO_3, evaporate, and ignite as above. Cool the dish in a desiccator and weigh.

6. *Protein*. Introduce a known weight of milk (5 to 10 g.) into a 500-ml. Kjeldahl digestion flask and add 20 ml. of concentrated sulfuric acid and

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[37] Lead or aluminum foil dishes (which are much cheaper) make a very satisfactory substitute for the platinum dishes.

[38] The percentage of total solids may be calculated from the specific gravity and percentage of fat by means of the following formula:

$$S = 0.25\,L + 1.2\,F$$

S = total solids. L = lactometer reading (third and fourth decimal places of specific gravity). F = fat content.

[39] Great care should be used in this ignition, the dish at no time being heated above a faint redness, since chlorides may volatilize.

about 0.2 g. of copper sulfate. Expel the major portion of the water by heating over a low flame, and finally use a full flame and allow the mixture to boil one to two hours. Complete the determination according to the directions given under the Kjeldahl Method, p. 874. If large amounts of milk are not available, a micro method may be used (see p. 880).

CALCULATION. Multiply the total nitrogen content by the factor 6.38[40] to obtain the protein content of the milk examined.

7. *Casein.* To 10.5 ml. (or a weighed equivalent) of fresh milk in a beaker, add 90 ml. of warm water (40° to 42° C.) and 1.5 ml. of 10 per cent acetic acid and stir. After 5 minutes' standing decant on an acid-washed filter, and wash the precipitate by decantation several times with cold water. Transfer the precipitate to the filter and repeat the washing twice. The filtrate should be clear or very nearly so. If the first portions are not clear, repeat the filtration and complete the washing of the precipitate. Determine the nitrogen content in the washed precipitate and filter, as directed in Exp. 6.

CALCULATION. Total N × 6.38 = casein content of aliquot.

8. *Hart's Casein Method.* Introduce 10.5 ml. of milk into a 200-ml. Erlenmeyer flask and add 75 ml. of distilled water and 1.0 to 1.5 ml. of 10 per cent acetic acid.[41] Mix the contents by giving the flask a vigorous rotary motion. The precipitated casein is now filtered off upon a 9- to 11-cm. filter paper.[42] Wash out the absorbed and loosely combined acetic acid by means of cold water. Continue the washing of both the casein on the filter and that adhering to the flask, until the wash water has reached a volume of at least 250 ml.

Now return the precipitate and paper to the original Erlenmeyer flask, add 75 to 80 ml. of neutral (carbon dioxide-free) water, 10 ml. of 0.1 N potassium hydroxide, and a few drops of phenolphthalein. Stopper the flask and shake it vigorously, by hand or machine, until the casein has been brought into solution.[43] Rinse the stopper with neutral (carbon dioxide-free) water and titrate the alkaline casein solution at once with 0.1 N hydrochloric acid until there is a disappearance of all red color.[44]

CALCULATION. Subtract the corrected[44] acid reading from the 10 ml. of alkali used. The difference is the percentage of casein in the milk. For example, if it takes 6.7 ml. of 0.1 N hydrochloric acid to titrate the alkaline solution to the end point and the check test was equivalent to 0.2 ml. of 0.1 N acid, the casein value would be obtained as follows: 10 − (6.7 + 0.2) = 3.1 per cent casein.

9. *Coagulable Protein.* Exactly neutralize the filtrate obtained under Exp. 7 with 10 per cent NaOH solution, add 0.3 ml. of 10 per cent acetic acid, and

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[40] The usual factor employed for the calculation of protein content of average or mixed foods from the nitrogen content is 6.25. It is based on the observation that proteins contain on the average 16 per cent nitrogen. The factor 6.38 is used to calculate the protein content from the total nitrogen, since the total protein constituents of milk have a mean nitrogen content of 15.7 per cent.

[41] In general 1.5 ml. of acetic acid gives a clear solution which filters nicely, but occasionally, when the milk has a low casein value, it is advisable to use less acetic acid.

[42] The process of filtration may be retarded through the packing of the casein mass upon the filter paper. In this case conduct a fine stream of cold water against the upper point of contact of filter paper and casein. By this means the casein precipitate is loosened and gathers in the apex of the filter. This procedure is very essential. It is not necessary to remove the casein which adheres to the interior of the flask.

[43] Solution is indicated by the disappearance of the white casein particles which would otherwise settle to the bottom of the flask.

[44] A check test should be run parallel with the entire determination. Even with special precautions as to neutrality, it is generally found that an acid check of 0.2 to 0.3 ml. will be obtained. This check titration should be *added* to the volume of acid used in titration.

heat on a steam bath until the protein is completely precipitated. Collect the precipitate on an acid-washed filter, wash with cold H_2O, and determine the nitrogen as directed under Exp. 6.

CALCULATION. Multiply the total nitrogen by the factor 6.38 to obtain the albumin and globulin content.

10. Lactose.[45] To about 350 ml. of water in a beaker add 20 g. of milk, mix thoroughly, acidify the fluid with about 2 ml. of 10 per cent acetic acid, and stir the acidified mixture continuously until a flocculent precipitate forms. At this point the reaction should be distinctly acid to litmus. Heat the solution to boiling for one-half hour, filter, rinse the beaker thoroughly, and wash the precipitated proteins and the adherent fat with hot water. Combine the filtrate and wash water and concentrate the mixture to about 150 ml. Cool the solution and dilute it to 200 ml. in a volumetric flask. Titrate this sugar solution according to directions given under Benedict's Method (see p. 919).

Myers recommends the following procedure for the determination of lactose in milk. One part of milk is mixed with an equal volume of phosphotungstic acid solution (70.0 g. of acid and 200 ml. of concentrated HCl in 1 liter of water) and 2 to 3 parts of water. Mix well, filter until clear, and titrate the clear filtrate against Benedict's solution (25 ml. reduced by 67 mg. of lactose).

BOCK'S METHOD FOR LACTOSE IN MILK. Pipet 20 ml. of milk into a 100-ml. volumetric flask. From a pipet or buret add 12 ml. of 10 per cent sodium tungstate and 12 ml. of $\frac{2}{3}$ N sulfuric acid. Mix by rotating the flask, dilute to the mark, and filter. Determine the lactose in the filtrate by titration with Benedict's solution.

CALCULATION. Make the calculation in the above titration methods, according to directions given under Benedict's Method (see p. 920), bearing in mind that 25 ml. of Benedict's solution are completely reduced by 0.067 g. of lactose.

11. Micro Method for Lactose in Milk.

PRINCIPLE. Lactose is determined on the protein-free filtrate of milk by a copper reduction method, following the method of Folin and Wu for blood sugar. This method is well suited to routine analyses.

Procedure. Introduce 1.0 ml. of milk into a 100-ml. volumetric flask, add 2 ml. of 10 per cent sodium tungstate. Add gradually 2 ml. of $\frac{2}{3}$ N sulfuric acid (or 16 ml. of N/12 acid), mix well, and let stand 5 minutes. Dilute to the mark with water and filter. Into a Folin-Wu sugar tube introduce 1 ml. of the filtrate and 1 ml. of water. Into another tube place 2 ml. of standard lactose solution.[46] Add 2 ml. of the Folin-Wu alkaline copper solution (see p. 568) to each tube, and heat in boiling water for 8 minutes. Cool and add 4 ml. of acid molybdate reagent (see p. 568) to each tube. After 1 minute, add diluted acid molybdate solution (1:4) to the 25-ml. mark, mix, and compare in the colorimeter.

CALCULATION.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.6 \times \frac{100}{0.01} \times \frac{1}{1000} = \text{Per cent lactose}$$

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[45] A method for lactose suitable for tissue analysis, or where other sugars may be present in significant amounts, is described by Malpress and Morrison: *Biochem. J.*, 45, 455 (1949).

[46] Prepare a stock standard solution by dissolving 1 g. of lactose in 0.2 per cent benzoic acid and making up to a volume of 100 ml. The working standard is prepared by diluting 3 ml. of the stock solution to 100 ml. with 0.2 per cent benzoic acid (2 ml. = 0.6 mg lactose).

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9

Epithelial and Connective Tissues: Bone and Teeth

EPITHELIAL TISSUE (KERATIN)

The major portion of hair, horn, hoof, feathers, nails, and the epidermal layer of the skin is made up of albuminoid proteins called keratins. As a class the keratins are characterized by their extreme insolubility in the usual protein solvents, their lack of digestibility, and their high sulfur content, most of which is in the form of cystine. These characteristics are not unrelated; the keratin molecule is considered to consist of closely packed polypeptide chains which are held together by the disulfide bond of cystine, the resistance to solvents and enzymes being associated with the close packing of the chains. This view is supported by the fact that if wool, for example, is ground to a fine powder by mechanical means it becomes more soluble and more digestible.

According to Block the various keratins may be further characterized as eukeratins and pseudokeratins. The eukeratins contain, in addition to other amino acids, the amino acids histidine, lysine, and arginine in the approximate ratio of 1:4:12, with from 3 to 5 per cent sulfur, nearly all of which is cystine. They are found largely in the hair, nails, feathers, horn, etc. The pseudokeratins, found chiefly in the skin and nervous tissue, contain less sulfur, from 1 to 3 per cent, and do not show the same histidine: lysine: arginine ratios as the eukeratins. It has been shown by x-ray crystallography that the intramolecular spatial arrangement of keratin can be modified under the influence of pressure or stretching. Such differences in molecular configuration of keratin may exist in the varied anatomic forms of epithelial tissue.

The keratins are not acted upon by proteolytic enzymes. This is perhaps due more to lack of physical accessibility to the proteolytic enzymes than to lack of specific types of peptide linkages. Hence if the molecular structure of keratin is changed through chemical or mechanical means these enzymes digest the resultant product quite readily. The keratins appear to be digestible by an enzyme in the intestine of the clothes moth at pH 9 in the presence of the reducing medium yielded by the H_2S liberated through bacterial action in the intestine.

The composition of human hair is influenced by its color and by the race, sex, age, and purity of breeding of the individual.[1] It may be differentiated from all other animal hair or wool by its high content of cystine.

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[1] Rutherford and Hawk: *J. Biol. Chem.*, 3, 459 (1907).

Most of the S of hair is in the form of cystine, with some cysteine usually present. Human hair contains from 13 to 19 per cent of cystine, sheep's wool 8 to 14 per cent, and feathers 7 to 12 per cent. It is claimed that the vitamins inositol, nicotinic acid, pantothenic acid, and riboflavin are present in normal rat and human hair in about the same ratio as in other tissues.[2]

From the analyses of the skin of the dog, rabbit, and man for mineral constituents, it has been shown that there is an individual variability in composition in the same species as well as distinct differences among different species. The addition of cystine to a diet deficient in this amino acid appears to favor the growth of hair to a greater extent than it affects the growth of body tissue as a whole.

Permanent waving is believed to rest upon a change in the structure of the keratin molecule. The damp heat used in the waving process opens up the disulfide bonds of the keratin. If the hair be artificially curled while in this condition, then dried, the disulfide bonds again form and the wave is automatically held in position. On the other hand if the disulfide groups are changed to sulfhydryl groups by reduction the permanent wave may be made without resorting to heat. Hence the so-called "cold" wave.

The addition of cystine to a sheep's diet will improve the weight and quality of the wool of the animal. In rats, para-aminobenzoic acid is also reported to have prevented the graying of hair. Such findings on lower animals have not been successfully duplicated in controlled observations on human beings.

Hyaluronic Acid and Hyaluronidase. So-called *mucopolysaccharides* occur in epithelial and connective tissues. The most important member of this group is *hyaluronic acid*. This may be hydrolyzed by the enzyme *hyaluronidase*.[3] This enzyme was originally termed the *spreading factor* as it was thought to be importantly related to the spread of disease germs in the body. It seems that the hyaluronic acid present in the intercellular substance offers resistance to the spread of harmful microorganisms. However, many such microorganisms manufacture hyaluronidase, which renders hyaluronic acid more or less impotent, permitting the harmful germ to enter the tissues. Thus in rheumatic fever it has been claimed that the therapeutic use of salicylates inhibits the action of hyaluronidase. The intact hyaluronic acid then acts to prevent invasion of the tissues.

In pure form, hyaluronidase, which is also present in spermatozoa, leech heads, and the venom of snakes and bees, has been used clinically to facilitate the hypodermic administration of large volumes of fluids (*hypodermoclysis*). To assist the penetration of certain drugs into mucous membranes, to combat the formation of renal calculi, to speed recovery from painful ankle sprains, and to aid in spreading the effect of local anesthetics over a larger area—these are other clinical functions ascribed to this enzyme. Evidence has accumulated which has cast some doubt upon the supposed efficiency of hyaluronidase in hypodermoclysis.[4]

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[2] Novak and Bergeim: *J. Biol. Chem.*, **155**, 283 (1944).

[3] Meyer: *Physiol. Revs.*, **27**, 335 (1947).

[4] *J. Am. Med. Assoc.*, **151**, 644 (1953).

EXPERIMENTS ON EPITHELIAL TISSUE (KERATIN)

Horn shavings or nail parings may be used in the experiments which follow:

1. ***Millon's Reaction.***
2. ***Xanthoproteic Reaction.***
3. ***Glyoxylic Acid Reaction (Hopkins-Cole).***
4. ***Test for Cystine and Cysteine Sulfur.***

What amino acids do these tests show to be present in keratin?

CONNECTIVE TISSUE

I. WHITE FIBROUS TISSUE

The principal solid constituent of white fibrous connective tissue is the albuminoid collagen. This substance is also found in smaller percentage in cartilage, bone, and ligament, but the collagen from the various sources is not identical in composition. In common with the keratins, collagen is insoluble in the usual protein solvents, presumably because of the close packing of the polypeptide chains, as with the keratins. It differs from keratin in containing much less sulfur. Analyses show only 0.1 per cent cystine and 0.9 per cent methionine. It contains no tryptophan and very little tyrosine. It has been estimated that glycine represents one-third of all the amino acid residues in the collagen molecule; a second third of the molecule is composed of proline and hydroxyproline, the other amino acids making up the remainder of the molecule. It is digested slowly by pepsin-HCl but by trypsin only at temperatures above 40° C. or after previous action of pepsin. One of the chief characteristics of collagen is the property of being converted by boiling acid or water to gelatin. The process does not seem to be one of hydrolysis since there is no increase in amino nitrogen. Some intramolecular change may occur, but the x-ray diagram does not change. This suggests that the alteration may be largely a physical one. The amino acid composition is essentially that of gelatin (see p. 122).

In vitamin C (ascorbic acid) deficiency (scurvy) the intercellular cement substances are not deposited in a normal manner. It is believed that in this condition there is a tendency to hemorrhage and to the slow healing of wounds because of the presence of abnormal collagen. In this connection Robertson[5] reports no significant differences in the collagen content of skin, liver, lung, kidney, spleen, costochondral junctions, and teeth of normal and acutely and chronically scorbutic guinea pigs. The same author[6] has shown that when guinea pigs in various nutritional states were fed glycine-N^{15} the collagen isolated from liver, lung, muscle, skin, and bone contained N^{15} in varying concentrations. The glycine isolated from the collagen of skin, muscle, and bone also contained an excess of the isotope. The concept of *collagen diseases* has received much attention. A wide variety of dissimilar diseases give definite evidence of fibrinoid changes.[7] Hence the clinician has been warned that no special emphasis should be placed upon the occurrence of these changes and the clinical usefulness of the term "collagen disease" is questioned.

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[5] Robertson: *J. Biol. Chem.*, **187**, 673 (1950).

[6] *Ibid.*, **197**, 495 (1952).

In the preparation of leather the collagen of the animal hide is purified and tanned. Certain precipitating agents such as tannic acid and the salts of heavy metals bring about the *tanning*.

The form of white fibrous tissue most satisfactory for general experiments is the tendo achillis of the ox. The fresh tissue has the following composition:

Water	62.9 per cent
Solids	37.1
Inorganic matter	0.5
Organic matter	36.6
Fatty substance (ether-soluble)	1.0
Coagulable protein	0.2
Mucoid	1.3
Elastin	1.6
Collagen	31.6
Extractives, etc	0.9

The mucoid just mentioned is called tendomucoid and is a glycoprotein. It possesses properties similar to those of other connective-tissue mucoids, e.g., osseomucoid and chondromucoid.

Gelatin, the substance which results from the treatment of collagen with boiling water or boiling dilute acids, is sometimes classed as an albuminoid. It is probably better to consider gelatin a protein derivative not properly belonging to any of the recognized classes of proteins. Gelatin differs from the keratins and collagen in having a much simpler physical structure and in being easily soluble and digestible. In fact a large part of a gelatin solution can pass through membranes such as the walls of capillaries, because of its small molecular weight. Gelatin is nonantigenic and is not a complete protein from the nutritional point of view, since it is lacking in tryptophan and low or lacking in certain other amino acids (see p. 122). Thus it is not satisfactory as the sole dietary protein, but because of its ease of digestion and absorption it is used as an accessory protein in the diet, particularly in the case of convalescents. Attempts to remedy the dietary deficiencies of gelatin by supplementing the diet with the missing amino acids have not been successful; the reason is not known. Gelatin gives a negative Hopkins-Cole test because it is lacking in tryptophan. The low content of tyrosine and cystine usually results in a negative or at the most a faintly positive reaction with Millon's reagent and the lead-blackening test. The isoelectric point of gelatin is about pH 4.7.

EXPERIMENTS ON WHITE FIBROUS TISSUE

The tendo achillis of the ox may be taken as a satisfactory type of the white fibrous connective tissue.

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[7] *J. Am. Med. Assoc.*, **150**, 220 (1952).

1. Preparation of Tendomucoid. Dissect away the fascia from about the tendon and cut the clean tendon into small pieces. Wash the pieces in running water, subjecting them to pressure in order to remove as much as possible of the soluble protein and inorganic salts. This washing is very important. Transfer the washed pieces of tendon to a flask and add 300 ml. of half-saturated lime water. Shake the flask at intervals for 24 hours. Filter off the pieces of tendon and precipitate the mucoid with dilute hydrochloric acid. Allow the mucoid precipitate to settle, decant the supernatant fluid, and filter the remainder. Test the mucoid as follows:

a. TEST FOR CYSTINE AND CYSTEINE SULFUR.

b. HYDROLYSIS OF TENDOMUCOID. Place the remainder of the mucoid in a small beaker, add about 30 ml. of water and 2 ml. of dilute hydrochloric acid, and boil until the solution becomes dark brown. Cool the solution, neutralize it with concentrated sodium carbonate, and test by Benedict's test.

2. Collagen. This substance is present in the tendon to the extent of about 32 per cent. Therefore in making the following tests upon the pieces of tendon from which the mucoid, soluble protein, and inorganic salts were removed in the last experiment, we may consider the tests as being made upon collagen.

a. BIURET TEST.

b. XANTHOPROTEIC REACTION.

c. GLYOXYLIC ACID REACTION (HOPKINS-COLE).

d. TEST FOR CYSTINE AND CYSTEINE SULFUR. Take a large piece of collagen in a test tube and add about 5 ml. of sodium hydroxide solution. Heat until the collagen is partly decomposed, then add 1 to 2 drops of lead acetate and again heat to boiling.

e. FORMATION OF GELATIN FROM COLLAGEN. Transfer the remainder of the pieces of collagen to a casserole, fill the vessel about two-thirds full of water, and boil for several hours, adding water at intervals as needed. By this means the collagen is transformed and gelatin is produced (see p. 245).

3. Gelatin. On the gelatin formed from the transformation of collagen in the above experiment (e), or on gelatin furnished by the instructor, make the following tests:

a. MILLON'S REACTION.

b. GLYOXYLIC ACID REACTION (HOPKINS-COLE).

c. TEST FOR CYSTINE AND CYSTEINE SULFUR.

Make the following tests upon a solution of gelatin in hot water:

a. COAGULATION TEST. Does it coagulate upon boiling?

b. PRECIPITATION BY ALCOHOL. Fill a test tube one-half full of 95 per cent alcohol and pour in a small amount of concentrated gelatin solution. Do you get a precipitate? How would you prepare pure gelatin from the tendo achillis of the ox?

II. YELLOW ELASTIC TISSUE (ELASTIN)

The ligamentum nuchae of the ox may be taken as a satisfactory type of the yellow elastic connective tissue. The principal solid constituent of this tissue is elastin, a member of the albuminoid group. In common with the keratins and collagen, elastin is an insoluble substance and gives the protein color reactions. It differs from keratin in amino acid composition and in the fact that it may be digested by proteolytic enzymes (see pp. 122 and 183–184). Elastin is characterized by its low sulfur content and its high content of leucine plus isoleucine.

Yellow elastic tissue also contains mucoid and collagen but these are present in much smaller amount than in white fibrous tissue, as may be seen from the following percentage composition[8] of the fresh ligamentum nuchae of the ox.

Water	57.6 per cent
Solids	42.4
Inorganic matter	0.5
Organic matter	41.9
Fatty substance (ether-soluble)	1.1
Coagulable protein	0.6
Mucoid	0.5
Elastin	31.7
Collagen	7.2
Extractives, etc	0.8

EXPERIMENTS ON ELASTIN

1. *Preparation of Elastin* (*Richards and Gies*). Cut the ligament into fine strips, run it through a meat chopper, and wash the finely divided material in cold, running water for 24 to 48 hours. Add an excess of half-saturated lime water, and allow the hashed ligament to extract for 48 to 72 hours. Decant the lime water, remove all traces of alkali by washing in water, and then boil in water with repeated renewals until only traces of protein material can be detected in the wash water. Decant the fluid and boil the ligament in 10 per cent acetic acid for a few hours. Treat the pieces with 5 per cent hydrochloric acid at room temperature for a similar period, extract again in hot acetic acid and in cold hydrochloric acid. Wash out traces of acid by means of water and then thoroughly dehydrate by boiling alcohol and boiling ether in turn. Dry in an air bath and grind to a powder in a mortar.

1. Millon's Reaction.

2. Xanthoproteic Reaction.

3. Biuret Test.

4. Glyoxylic Acid Reaction (Hopkins-Cole).

5. Test for Cystine and Cysteine Sulfur.

III. CARTILAGE

The principal solid constituents of the matrix of cartilaginous tissue are chondromucoid, chondroalbumoid, and collagen. Chondromucoid on decomposition yields protein and chondroitin sulfuric acid which has the following formula:

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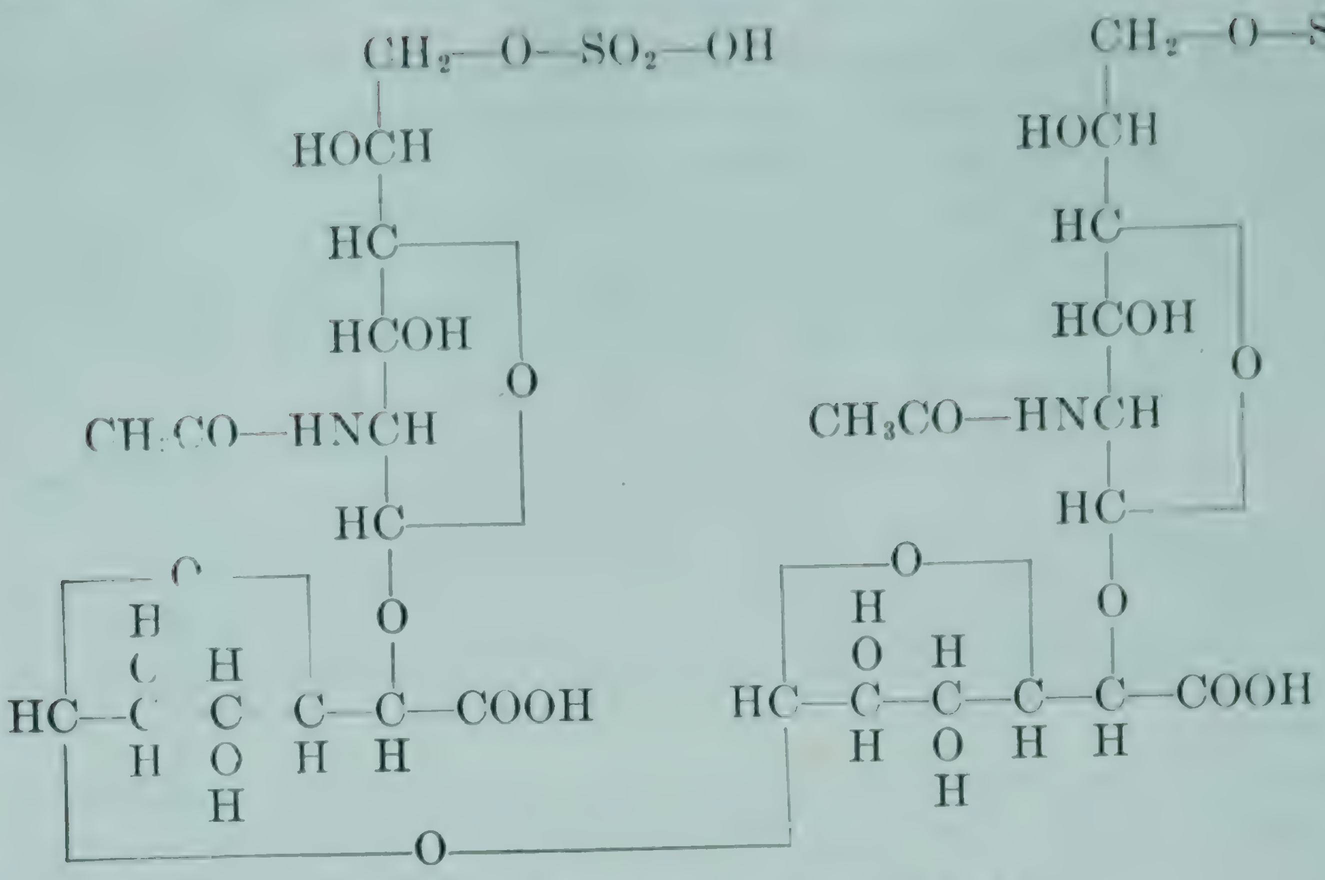
[8] Later experiments (Hall, Reed, and Tunbridge: *Nature*, **170**, 264 (1952)) show the presence of *elastomucin*, a carbohydrate-containing protein. The "elastin" of the earlier investigators was probably a mixture of elastin and elastomucin.



Chondroitin sulfuric acid

On hydrolysis it loses sulfuric acid and forms chondroitin. The latter loses acetic acid to form chondrosin. Chrondrosin on hydrolysis yields chondrosamine $CH_2OH(CHOH)_3CHNH_2CHO$ and glucuronic acid $COOH(CHOH)_4CHO$. Chondrosamine is apparently a galactosamine. The amino sugars are dealt with differently in metabolism from glucose or simple amino acids. They apparently do not form glycogen in the body. Chondromucoids in various tissues differ from each other in the character of the protein only. Chondroitin sulfuric acid differs from the sulfuric acids found in the mucin of saliva, etc., in that the carbohydrate group in the latter is chitosamine (probably a glucosamine). It is of interest that chitin in the exoskeleton of certain lower animals is a polysaccharide containing chitosamine and acetic acid. Chondroitin sulfuric acid is found in tendomucoid and apparently also in osseomucoid, which are thus closely related to chondromucoid.

It has been shown that a deposition of radioactivity in the knee-joint cartilage of young rats follows the administration of radioactive sulfur in the form of sulfate. This radioactivity was concentrated in the chondroitin sulfate (chondroitin sulfuric acid) fraction of the cartilage. Thiouracil and thyroxine influence the radioactivity pattern. For example, in animals pretreated with thiouracil the uptake of radioactive sulfur in the cartilage was reduced. The administration of thyroxine counteracted the action of thiouracil. It was further shown through the use of isotopes that 0.134 g. of chondroitin sulfate was formed per 100 g. of cartilage in 24 hours. The actual rate of sulfate formation was thus determined for the first time.

Chondroalbumoid is similar in some respects to elastin and keratin. It differs from keratin in being digestible by proteolytic enzymes and in containing considerably less sulfur than any member of the keratin group. It gives the usual protein color reactions.

EXPERIMENTS ON CARTILAGE

1. *Preparation of Chondroitin Sulfuric Acid from Chondromucoid.* Free nasal septa of cattle from bone and other extraneous material. Run 200 g. of the material through a meat chopper. Add 400 ml. of 2 per cent NaOH. Let stand for 2 days. Strain through cloth. Treat the residue again with 200 ml. of NaOH for 2 days. Strain. Wash the residue once with water. Combine the extracts. Acidify with acetic acid and then add an excess of barium carbonate. Concentrate on the steam bath to half the volume. Pour off the clear liquid. Filter the remainder on a folded filter and add filtrate to the decanted solution. Acidify. Evaporate to about 80 ml. Centrifuge to remove protein and barium carbonate. Drop the clear yellow liquid into 8 volumes of glacial acetic acid kept vigorously agitated (preferably with a turbine). Filter off the acid potassium salt with suction. Wash with glacial acetic acid and then with alcohol and ether.

Dissolve 8 g. of the product which still gives a slight biuret test in 400 ml. of water, and while the solution is kept stirred drop in basic lead acetate solution to complete precipitation. Wash the precipitate several times by rubbing in a mortar with water and filtering with suction. Dissolve the precipitate in 10 per cent HCl. To the filtrate from the lead chloride add glacial acetic acid to precipitate all of the chondroitin sulfuric acid. Wash with glacial acetic acid, alcohol, and finally ether.

2. *To Show the Presence of Sulfuric Acid and of Reducing Sugars in Chondromucoid.* Treat 50 g. of cartilage from nasal septum of the ox or cartilage rings from trachea of the ox with 100 ml. of 1 per cent NaOH and let stand over night. Pour off 50 ml. of fluid, add 5 ml. of concentrated HCl and boil for 30 minutes, bringing down to a low volume. To one portion add $BaCl_2$ solution and note the precipitate of $BaSO_4$. Neutralize another portion with sodium carbonate and apply Benedict's test to show the presence of a reducing carbohydrate group.

OSSEOUS TISSUE (BONE)

Bone contains from 14 to 44 per cent water, depending upon the type of bone and its location in the body. Of the fat-free dry matter of bone, from 30 to 50 per cent is organic, and the remainder inorganic. Typical analyses of bone are given in Chapter 35 in connection with the discussion of rickets and vitamin D.

The organic portion of bone is similar in composition to cartilage. It contains collagen (*ossein*), osseoalbumoid, and osseomucoid. These proteins resemble the corresponding proteins in cartilage and tendon. Because of the collagen content, gelatin is formed when bone is boiled with dilute acid. The bone marrow also contains fat.

The inorganic material of bone consists chiefly of calcium, phosphate, and carbonate with small amounts of magnesium, sodium, strontium, lead, citrate, fluoride, hydroxide, and sulfate. These substances are present in amounts which correspond to 84 per cent $Ca_3(PO_4)_2$, 10 per cent $CaCO_3$, 2 per cent $Ca_3(\text{citrate})_2$, 1.0 per cent $Mg_3(PO_4)_2$, 1.0 per cent $MgCO_3$, and 2.0 per cent Na_2HPO_4. This composition is by no means constant. Wide variations have been encountered. For example, the molar proportions of $PO_4:CO_3$ can be anywhere from 1.8 to 3.4. These changes in composition are related to changes in the $PO_4:CO_3$ ratio of blood serum. The composition of blood serum in turn is influenced by nutrition, age, and disease.[9] Despite the wide variations encountered in composition, x-ray diffraction studies reveal only one crystal structure, namely the *apatite lattice*. These hexagonal crystals in the bone are small in size, the dimensions seen in the electron microscope being about 500 × 250 × 100 Ångstrom units (Fig. 69).

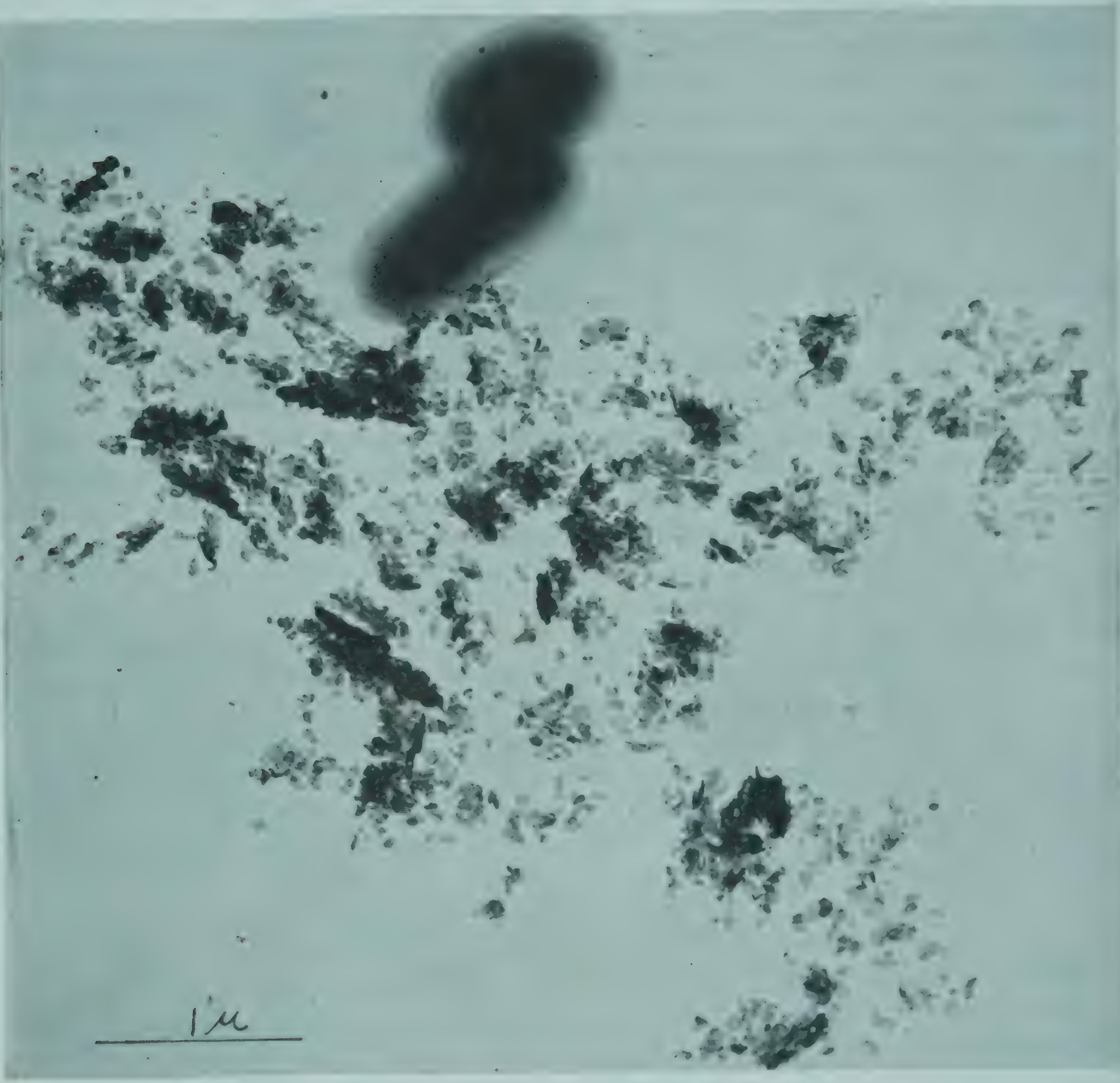


FIG. 69. MICROGRAPH OF CORTICAL BONE.

The diaphysis of a cat's femur autoclaved, agitated in blendor, and subjected to ultrasonics. The two bacteria are *Bacillus aerogenes* and give an idea of the relative size of the crystals in relation to bacteria. Magnification 34,500.

Courtesy, R. A. Robinson and F. W. Bishop, and *Science*.

Because of this small size, the bone crystal offers a large surface that can adsorb other compounds than those which account for the crystal lattice itself. Evidence for this theory was the preferential solubility of carbonates and citrates in bone. There are many alternative theories, but the one which appears to be most acceptable is that of Hendricks, who visualizes the crystal structure of bone salts as due to hydroxyapatite

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[9] Sobel, Rockenmacher, and Kramer: *J. Biol. Chem.*, 158, 475; and 159, 159 (1945).

$Ca_{10}(PO_4)_6(OH)_2$, in which F may replace OH, with the rest of the composition held by surface forces (Fig. 70).

Despite the possible variation in bone composition indicated in the discussion above, under some conditions the inorganic material of bone is rather constant in composition. Observations on the constancy of composition of bone during fasting are of interest in this connection. The percentage composition of the dry fat-free femurs of two dogs, after the animals had fasted for 104 and 14 days respectively, was as follows:

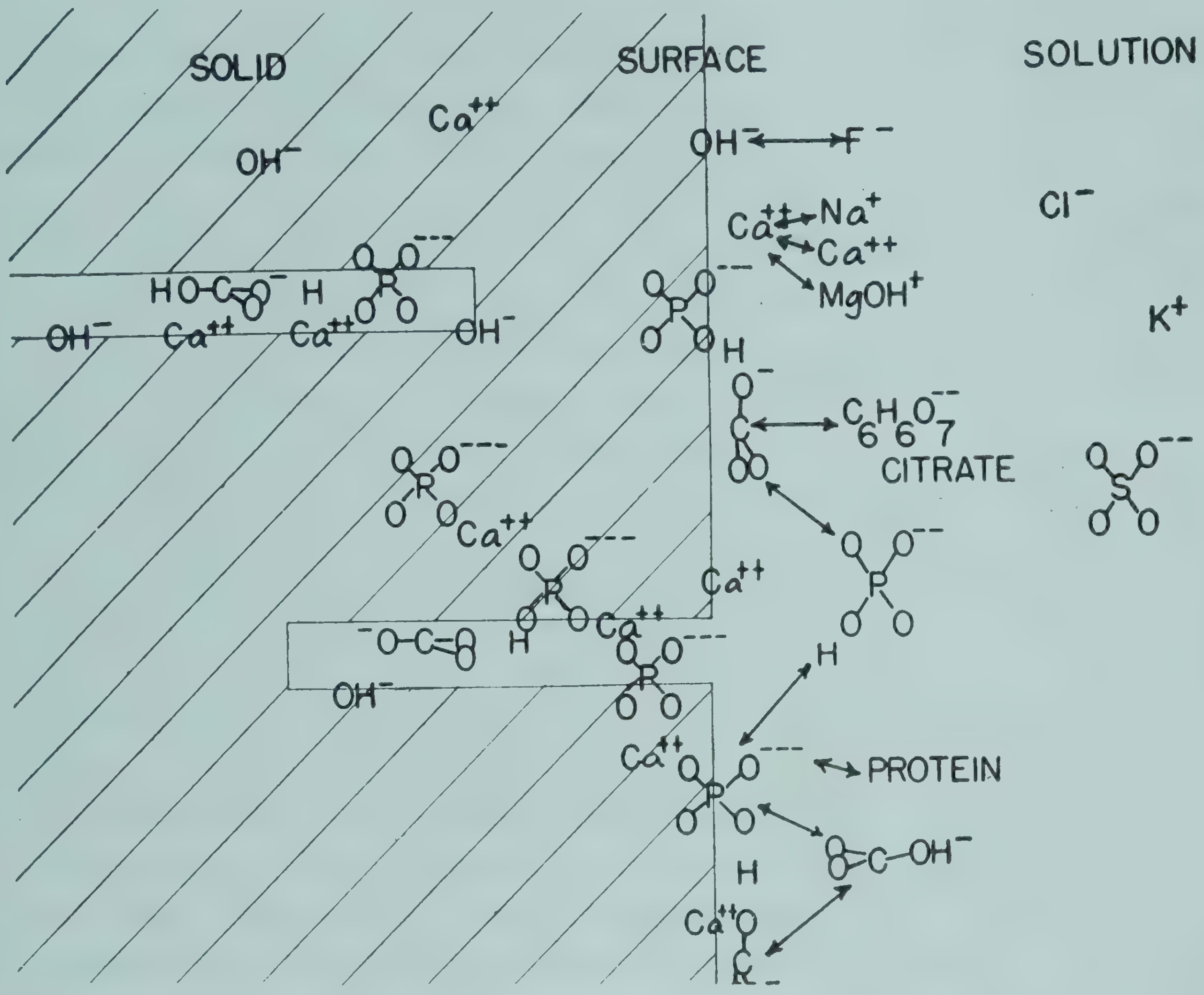

FIG. 70. SCHEMATIC INDICATIONS OF SURFACE COMPOUND FORMATION IN THE BONE SALT.

Courtesy, S. B. Hendricks, "Comments on Crystal Chemistry of Bone" from the *Fourth Conference on Metabolic Interrelations* of the Josiah Macy, Jr. Foundation.

Dog No.	*Length of Fast*	*Ash*	*N*	*Ca*	*Mg*	*P*
1	104 days	61.5	4.6	23.3	0.5	12.8
2	14 days	61.7	4.1	23.2	0.5	12.9

The marked uniformity in composition notwithstanding the wide variation in the fasting periods is significant. The tensile strength of the femur of the dog has been found to be at least 25,000 pounds to the square inch whereas that of oak is 10,000 and that of cast iron 20,000 pounds to the square inch.

The percentage compositions of normal human bone and of bone from a case of osteomalacia with respect to certain elements are given in the following table:

Constituent	*Kind of Bone*	
	Normal	*Osteomalacia*
Calcium	20.2	10.8
Magnesium	0.1	0.3
Phosphorus	8.6	5.3
Sulfur	0.1	0.6

Hammett has shown in the case of albino rats that there is an increase in calcium and a decrease in magnesium and phosphorus during the first 75 days of growth. Moreover the bones of the female have a higher calcium content than the bones of the male.

Chemistry of Ossification. Evidence from many sources indicates that there is an active metabolism in bone. The mineral matter of bone may be drawn upon in case of need elsewhere, as in the formation of milk by the lactating animal and in the formation of the bones of the fetus during pregnancy. This utilization of bone material should be regarded as a resorption of bone rather than a simple decalcification since both the organic and inorganic components of bone disappear during the process. Studies with radioactive phosphorus as a tracer[10] have shown that almost immediately after the introduction of radioactive phosphorus into the blood there is an appreciable uptake of the labeled phosphorus by the bones, indicating a turnover or metabolic interchange between the bone and plasma or lymph phosphorus. During a 50-day period in the life of the rabbit, for example, half the scapula may be replaced, with a 30 per cent turnover of the epiphyseal phosphorus; diaphyseal bone metabolism appears to be considerably slower. There are indications of a labile fraction of bone which is in equilibrium with the plasma and a stable fraction which is in equilibrium with the labile portion.

The mechanism of *calcification* is not clear. That the solubility-product principle operates is indicated from studies of healing rickets in vivo as well as in vitro. Not until the product of the ionic concentration of calcium and phosphate exceeds a minimum is there any new mineralization in the embryonic or rachitic bone cartilage matrix. This minimum is not the same for all bones, as is shown in the table below:[11]

THE SOLUBILITY-PRODUCT
PRINCIPLE AND NEW
CALCIFICATION

Bone	*Ca × P**
Embryonic	16
Rachitic	35
Be Rickets	60
Sr Rickets	90

* Minimum for new calcification in vitro; *Ca* and *P* are expressed as mg. per 100 ml. of solution; *P* represents inorganic phosphate.

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[10] Hevesy: *Ann. Rev. Biochem.*, 9, 641 (1940); Cohn, Cohn, and Aub: *Ann. Rev. Biochem.*, 11, 415 (1942).

[11] Sobel and Hanok: *J. Biol. Chem.*, 197, 669 (1952).

Thus one must postulate the existence of local factors in the calcifying site whose concentration determines the level at which the solubility product operates. Several explanations have been offered for the nature of this local factor. Phosphatase, present in bone cartilage, has been suggested as the enzyme which on splitting organic phosphate causes supersaturation of calcium phosphate, thus promoting precipitation. It has also been suggested that glycogen and the enzymes responsible for phosphorylative glycogenolysis provide both the organic phosphate and the local mechanism. More recently, however, Sobel advanced the view that a mucopolysaccharide, chondroitin sulfate, is the local factor, based on (1) the presence of this compound at the site of mineralization; (2) the observation that when phosphatase and enzymes of the phosphorylative glycogenolysis are destroyed by heating and other measures, bone sections will still mineralize; (3) studies of the reversible inactivation of the ability of bone to calcify in vitro, by cations such as Be^{++} and Cu^{++} as inactivators and Ca^{++} as the reactivating ion.

Although the precipitate found in bone is composed of $[Ca_3(PO_4)_2]_n \cdot CaCO_3$ (n = 1.8 to 3.4), studies of the physicochemical mechanism indicate that the first aggregate formed is $CaHPO_4$ which is rapidly transformed to hydroxyapatite. So far, however, no direct evidence has been found for the existence of $CaHPO_4$ in bone.

Vitamin D promotes calcification, especially when added to rachitogenic diets. Under the influence of the antirachitic vitamin, the serum Ca × P product becomes elevated as shown below:

INFLUENCE OF VITAMIN D ON SERUM CA AND P*
(Mean Values)

Diet, per cent		*Serum, mg. per cent*			
Ca	*P*	*Ca*	*P*	*Ca*†	*P*†
1.20	0.12	11.7	2.1	13.3	3.4
0.19	0.12	9.4	4.7	11.1	6.0
0.03	0.76	5.6	7.5	8.8	8.4

* 23-day-old rats on a rachitogenic diet for 30 days.
† Receiving 100 units of vitamin D daily.
P = inorganic phosphate.

This elevation in serum Ca × P is probably the main reason for the effectiveness of vitamin D. In diseases like Fanconi's rickets in which this elevation of serum Ca × P does not take place, the rachitic condition persists. Though the mode of action of vitamin D is not fully clear, there is both increase in the absorption of calcium from the intestinal tract and increased resorption of phosphate from the kidney. Both of these effects of vitamin D would tend to elevate serum Ca × P and thus promote calcification. The finding of a high phosphatase content of the blood in osteitis deformans, rickets, and other generalized bone disorders, and a decrease of phosphatase in the tissues of the animals fed a diet high in vitamin D, are of interest in this connection. Calcium metabolism and deposition are also influenced by the endocrine glands (see Chapter 26, Hormones).

EXPERIMENTS ON OSSEOUS TISSUE

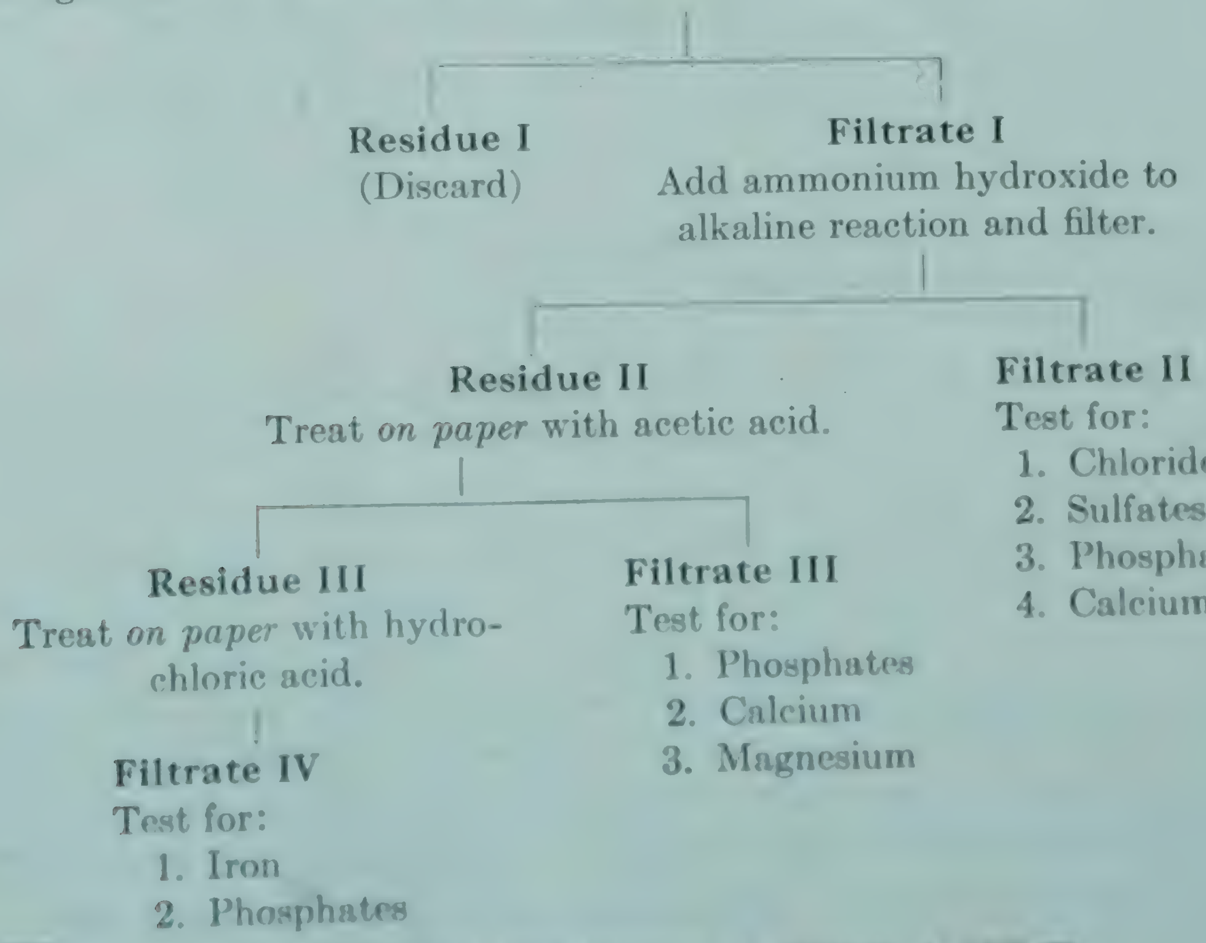
1. ***Decalcification of Bone.*** **Treat a strip of rib bone with dilute HNO_3 and permit it to soak for 6 to 8 days, renewing the acid every 1 to 2 days. After complete decalcification, wash the bone, split it, and remove the marrow. Now cut the bone into small pieces, wash it to remove acid, and boil the material in water for at least one hour. Filter, concentrate the filtrate, and permit it to cool. What is the characteristic of the cooled filtrate? What has been produced from the bone?**

2. ***Qualitative Analysis of Bone Ash.*** **Take 1 g. of bone ash in a small beaker and add a little dilute nitric acid. What does the effervescence indicate? Stir thoroughly, and when the major portion of the ash is dissolved add an equal volume of water and filter. To the acid filtrate add ammonium hydroxide to alkaline reaction. A heavy white precipitate of phosphates results. (What phosphates are precipitated here by the ammonia?) Filter and test the filtrate for chlorides, sulfates, phosphates, and calcium. Add dilute acetic acid to the precipitate on the paper and test a little of this filtrate for calcium and phosphates. Heat the remainder of the filtrate to boiling and add $(NH_4)_2CO_3$ and NH_4Cl slowly to this hot solution as long as a precipitate forms. Filter off the precipitate of $CaCO_3$ and wash with hot water until free from alkali.[12] To the filtrate add a solution of Na_2HPO_4, make strongly alkaline with NH_4OH, and note the formation of a white precipitate of ammonium magnesium phosphate (NH_4MgPO_4). Examine the crystals under the microscope and compare with those shown in Fig. 226. To the precipitate on the filter paper, which was insoluble in acetic acid, add a little dilute hydrochloric acid and test this last filtrate for phosphates and iron.**

Reference to the scheme below may facilitate the analysis.

Bone Ash

Add dilute nitric acid, stir thoroughly, and after the major portion of the ash has been brought into solution, add a little distilled water and filter.



Residue I
(Discard)

Filtrate I
Add ammonium hydroxide to alkaline reaction and filter.

Residue II
Treat *on paper* with acetic acid.

Filtrate II
Test for:
1. Chlorides
2. Sulfates
3. Phosphates
4. Calcium

Residue III
Treat *on paper* with hydrochloric acid.

Filtrate III
Test for:
1. Phosphates
2. Calcium
3. Magnesium

Filtrate IV
Test for:
1. Iron
2. Phosphates

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[12] Magnesium is not precipitated here because of presence of NH_4Cl.

MICROESTIMATION OF THE INORGANIC CONSTITUENTS OF BONE.[13] Sobel et al., have described a scheme for analysis of CO_3, Ca, P, and total base which is serial in operation and requires only one weighed specimen. The complete analysis can be performed on as little as 5 mg. of sample.

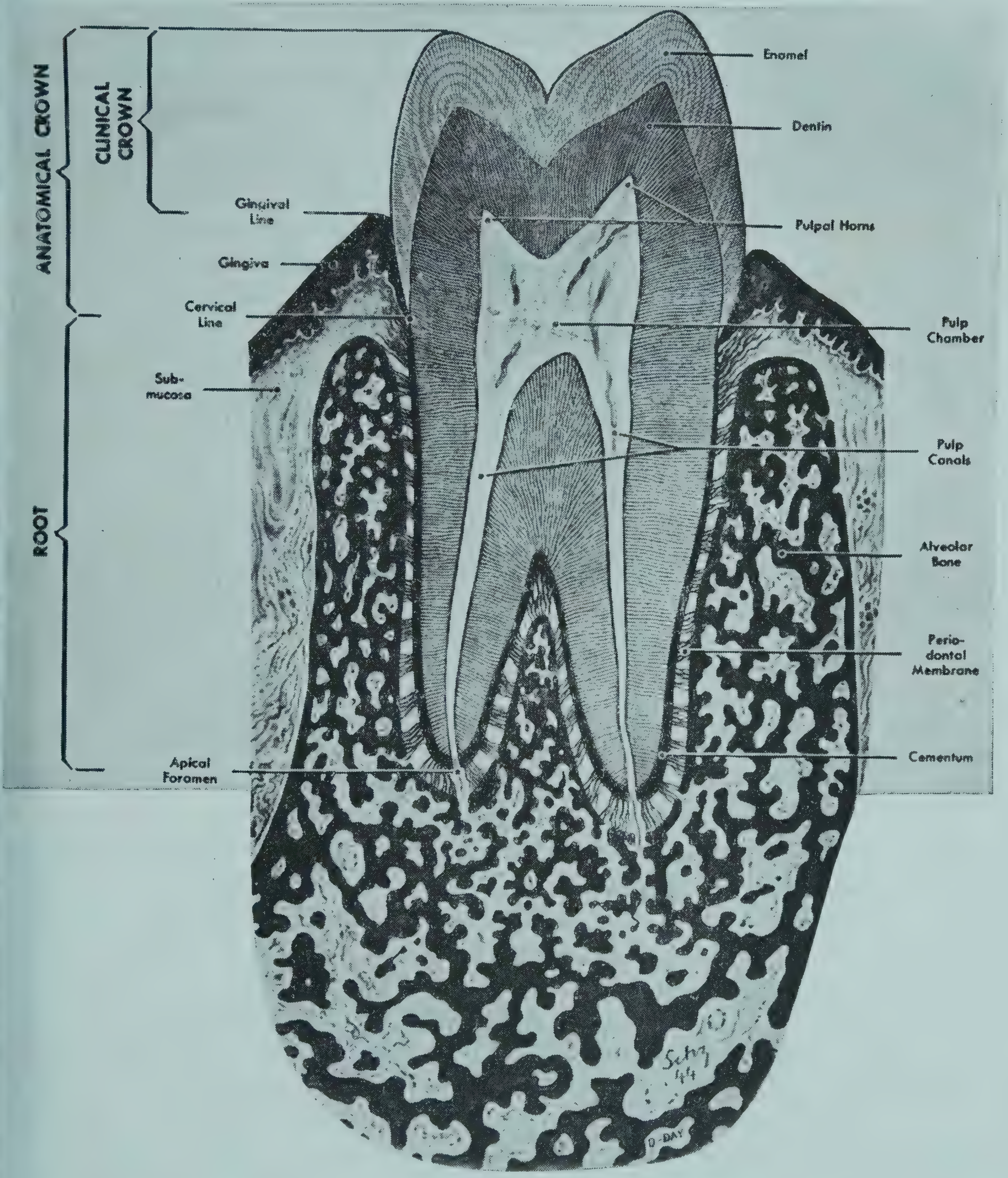
ANATOMICAL CROWN
CLINICAL CROWN
ROOT
Enamel
Dentin
Gingival Line
Pulpal Horns
Gingiva
Cervical Line
Pulp Chamber
Sub-mucosa
Pulp Canals
Alveolar Bone
Periodontal Membrane
Apical Foramen
Cementum
Schz 44
D-DAY

FIG. 71. SCHEMATIC DRAWING OF A TOOTH AND THE SURROUNDING STRUCTURE. Courtesy, Zeisz and Nuckolls: *Dental Anatomy*, St. Louis, C. V. Mosby Co., 1949, p. 25.

TEETH

Composition. Teeth are composed of four tissues: enamel, dentin, and cementum which are highly calcified, and the dental pulp which is uncalcified and is surrounded by the dentin (Fig. 71). Enamel covers the dentin in the crown of the tooth. It is the hardest substance in the body and contains the smallest amount of water. Cementum contains about 30 per cent organic matter. It covers the dentin in the root of the tooth, which is contained in the supporting structures known as the periodontal tissues. These tissues, which include the gingiva and alveolar bone, hold the teeth in line. The periodontal membrane, which is adjacent to the cementum, connects the teeth to the gingiva and alveolar bone.

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[13] Sobel, Rockenmacher, and Kramer: *J. Biol. Chem.*, **152**, 255 (1944).

The average composition of human enamel and dentin is shown in the following table.

COMPOSITION OF HUMAN ENAMEL AND DENTIN

Constituent	*Enamel*	*Dentin*
	*Per Cent on Dry Basis**	
Ash	97	72
Calcium	35.8	26.5
Magnesium	0.4	0.8
Sodium	0.7	0.2
Potassium	0.3	0.07
Phosphorus	17.4	12.7
Carbon dioxide (from carbonate)	2.8	3.06
Chlorine	0.3	0.03
Fluorine	0.0112	0.0204
Iron	0.0218	0.0072
Organic matter	1	20

* Water which is not removed at about 100° C., the temperature to which the material is usually heated before analysis, was included in the weight from which calculations were made. The percentage of such combined water is approximately 2 for enamel and 8 for dentin.

The average values for the composition of whole human teeth are shown in the following table.

Whole Human Teeth	
*Constituents in Per Cent**	
Calcium	29.7
Magnesium	0.6
Phosphorus	14.2
Carbon dioxide (from carbonate)	2.9
Water	8.7
Inorganic matter	84.7
Organic matter	15.3

* Data of Lefevre and Hodge: *J. Dental Research*, **16**, 279 (1937) and Crowell, Hodge, and Line: *J. Dental Research*, **14**, 251 (1934), recalculated. The value for water represents the loss in weight after heating the teeth at 96° C. for seven days. The weight after this treatment is the dry weight. The values for inorganic and organic matter, and the values for calcium, magnesium, phosphorus, and carbon dioxide represent the percentages of the dry weight (weight on dry basis).

On the basis of chemical analysis, it was thought at one time that most of the calcium and phosphorus in the dental tissues existed as $Ca_3(PO_4)_2$, a view formerly held also for bone. However, x-ray crystallographic studies suggest that the crystal structure of most of the calcium phosphate in the dental tissues is similar to that of hydroxyapatite, $Ca_{10}(PO_4)_6 \cdot (OH)_2$. The striking similarity of x-ray diffraction photographs of hydroxyapatite and enamel is shown in Fig. 72, which contains also the photographs of fluoapatite and chloroapatite. There is some evidence[14] which suggests that in the case of dentin and cementum additional phosphorus in the form of either $PO_4^{\equiv}$ or $HPO_4^{=}$ or some other ion involving phosphorus is occluded in the hydroxyapatite molecule. The apatite salt known as tricalcium phosphate hydrate, which has been claimed by some to be the main inorganic salt in dentin and cementum, is probably hydroxyapatite with occluded phosphate ion. It is claimed that smaller amounts of carbonateapatite, chloroapatite, and fluoapatite are present in enamel, and that dentin contains some fluoapatite. A portion of the sodium, potassium, and magnesium in the dental tissues may be part of the apatite lattice by substitution for part of the calcium. Enamel and dentin on spectrographic examination have been found to contain numerous trace elements, including aluminum, barium, boron, chromium, copper, lead, lithium, manganese, nickel, silicon, silver, strontium, tin, titanium, vanadium, and zinc.

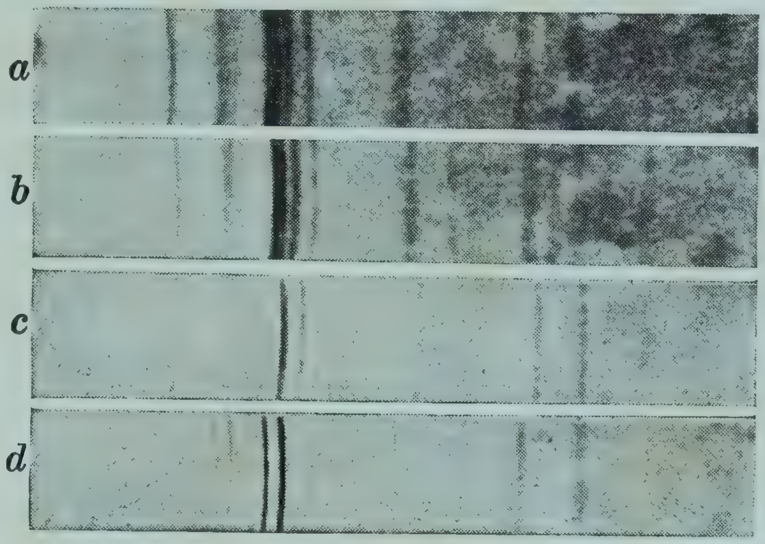

FIG. 72. X-RAY-DIFFRACTION PHOTOGRAPHS.
(*a*) Human dental enamel, (*b*) hydroxyapatite, (*c*) fluoapatite, (*d*) chloroapatite.
From Thewlis, Glock, and Murray: *Trans. Faraday Soc.* **35,** 358 (1939).

The electron microscope as well as x-ray studies reveal that the crystals of enamel are larger than those of bone or dentine.

The composition of growing teeth can be influenced in a predictable manner, high-carbonate teeth being produced on a high-calcium, low-phosphate diet and low-carbonate teeth on a low-calcium, high-phosphate diet.[15] Evidence to date indicates that high-carbonate teeth are more susceptible to caries.[16]

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[14] Posner and Stephenson: *J. Dental Research,* **31,** 371 (1952).
[15] Sobel and Hanok: *J. Biol. Chem.,* **176,** 1103 (1948).
[16] Sobel: *Fourth Josiah Macy Conference on Metabolic Interrelations,* 1952, p. 261.

The organic matter of enamel consists mainly of the protein keratin, which on the basis of the proportion of histidine, lysine, and arginine is of eukeratin nature (see p. 184). Small amounts of cholesterol and phospholipide are also present. The albuminoids collagen and elastin are found in dentin, collagen representing the major organic constituent of this tissue. Glycoprotein is present in dentin along with small amounts of cholesterol and phospholipide. Collagen is the main organic constituent of cementum. Citrate has been identified as a constituent of human teeth, five to eight times more being present in dentin than in enamel.

CHRONOLOGY OF HUMAN DENTITION
(Logan and Kronfeld, slightly modified by McCall and Schour)

Tooth		*First evidence of calcification*	*Enamel completed*	*Root completed*
Deciduous dentition	Central incisor	4 mos. in utero	4 mos.	1½–2 yrs.
	Lateral incisor	4½ mos. in utero	5 mos.	1½–2 yrs.
	Cuspid	5 mos. in utero	9 mos.	2½–3 yrs.
	First molar	5 mos. in utero	6 mos.	2–2½ yrs.
	Second molar	6 mos. in utero	10–12 mos.	3 yrs.
Permanent dentition				
Upper jaw	Central incisor	3–4 mos.	4–5 yrs.	10 yrs.
	Lateral incisor	1 yr.	4–5 yrs.	11 yrs.
	Cuspid	4–5 mos.	6–7 yrs.	13–15 yrs.
	First bicuspid	1½–1¾ yrs.	5–6 yrs.	12–13 yrs.
	Second bicuspid	2–2¼ yrs.	6–7 yrs.	12–14 yrs.
	First molar	At birth	2½–3 yrs.	9–10 yrs.
	Second molar	2½–3 yrs.	7–8 yrs.	14–16 yrs.
	Third molar	7–9 yrs.	12–16 yrs.	18–25 yrs.
Lower jaw	Central incisor	3–4 mos.	4–5 yrs.	9 yrs.
	Lateral incisor	3–4 mos.	4–5 yrs.	10 yrs.
	Cuspid	4–5 mos.	6–7 yrs.	12–14 yrs.
	First bicuspid	1¾–2 yrs.	5–6 yrs.	12–13 yrs.
	Second bicuspid	2¼–2½ yrs.	6–7 yrs.	13–14 yrs.
	First molar	At birth	2½–3 yrs.	9–10 yrs.
	Second molar	2½–3 yrs.	7–8 yrs.	14–15 yrs.
	Third molar	8–10 yrs.	12–16 yrs.	18–25 yrs.

Several investigations have been made in an attempt to ascertain whether differences in chemical composition exist between enamel of sound teeth and the intact enamel of carious teeth. No significant differences were found for the contents of calcium, magnesium, phosphorus, and carbonate. Similar studies on dentin have likewise given negative results. On the other hand, it has been reported[17] that enamel from sound teeth contains significantly more fluorine than does the sound enamel from carious teeth, although no difference was found for dentin. However, this difference between sound and carious teeth was not found in all localities where these determinations were made.[18] (See p. 262 for additional data on the relationship of fluorine and teeth.) No difference was found in the ash, calcium, and phosphorus contents of the root dentin of teeth from pregnant and nonpregnant women; this fact suggests that there is no basis for the view that minerals are withdrawn from the teeth during pregnancy. Furthermore, clinical investigation has shown that the incidence of dental decay in pregnant women is no greater than in nonpregnant women of corresponding age.

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[17] Armstrong and Brekhus: *J. Dental Research*, 17, 393 (1938).

Chronology of Dentition. The ages at which the deciduous and permanent teeth begin to calcify and are completed are shown in the table[19] on p. 258.

In a study of calcification of teeth at birth, it was found that all the teeth of both jaws contained approximately 212 mg. of calcium and 106 mg. of phosphorus. At the time of birth the lateral incisors, the cuspids, the first deciduous molars, the second deciduous molars, and the first permanent molars contained respectively 20, 7.5, 21, 11, and 0.1 per cent of the calcium present in the fully erupted teeth. These data thus indicate that the postnatal period is of far greater importance than the prenatal period in the calcification of the deciduous as well as of the permanent teeth.

SYSTEMIC EFFECTS

In studies dealing with the response of the dental tissues to systemic influences it is important to distinguish between the developing and the adult teeth, and between the dental tissues of limited growth, as in humans, and those of continuous growth like the incisors of rats. A great deal of confusion has been caused by failure to recognize this distinction.

Adult teeth of limited growth are probably only slightly affected by systemic influences. That such effects may occur is suggested by studies in which radioactive isotopes have been used. For example, it was shown that following the ingestion or injection of sodium phosphate containing radioactive phosphorus, small amounts of the isotope appeared in the enamel and dentin, but much smaller amounts were present in the former than in the latter. However, the slight extent to which this occurs is shown by the fact that after the ingestion of 900 mg. of sodium phosphate containing isotopic P by an individual twenty-five years of age, about 1/300,000 of the labeled P entered a single tooth. On the basis of such data it was calculated that the replacement of 1 per cent of the total tooth phosphorus by that taken up in food would take about 250 days.

For the proper calcification of the teeth the diet must contain adequate quantities of calcium and phosphorus and certain vitamins. Some of the hormones likewise play a fundamental role.

Vitamins, Calcium, and Phosphorus. The presence of vitamin A is essential to the process of tooth formation. Dietary deficiency of this vitamin during the period that the teeth are undergoing development causes the ameloblasts (enamel-forming cells) to atrophy, resulting in hypoplastic (incompletely developed) enamel which is imperfectly calcified. There is also atrophy of the odontoblasts (dentin-forming cells) so that the dentin laid down is also incompletely calcified. It is claimed that the primary effect is on the enamel, irregularities in the development of the dentin being secondary to the enamel hypoplasia. Deficiency of vitamin A in rats has been found to produce an increase in the percentage of magnesium in the incisors in spite of the fact that the ash content is decreased. A characteristic effect of vitamin A deficiency—namely, the substitution of stratified keratinizing epithelium for normal epithelium—has been observed in the gingiva. Malformation of alveolar bone has been produced in dogs by feeding them vitamin A-deficient diets.

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[18] McClure: *J. Dental Research*, **27**, 287 (1948).

[19] Logan and Kronfeld: *Histopathology of the Teeth and Their Surrounding Structures*, revised and re-edited by Boyle, Philadelphia, Lea & Febiger, 1949, p. 41.

Vitamin C is also important for the functional activity of the formative cells. Dietary deficiency in vitamin C during the period of tooth development causes impairment of the odontoblasts, leading to defective calcification of the dentin accompanied by hemorrhagic and degenerative lesions in the pulp tissue. The changes in the dentin and pulp have been attributed to an inability to produce and maintain intercellular substance. Defective enamel formation, which has also been observed, is said to be secondary to retarded dentin deposition. Pathological changes in gingival tissue have been demonstrated to be due to deficiency of vitamin C in the diet. These conditions, as well as those in the teeth themselves, have been shown to respond favorably to the addition to the diet of vitamin C.

The degree of mineralization of the teeth depends on the relative as well as on the absolute quantities of calcium and phosphorus in the diet, and also on the amount of vitamin D or exposure to ultraviolet rays. Even with optimal quantities of calcium and phosphorus the presence of vitamin D is essential to the development of perfect teeth. Hypoplastic defects in the enamel and imperfect calcification of the dentin have been found in rickets in children. It has been reported that an increase in the vitamin D intake brings about a reduction in the incidence of dental decay in children. Similar results have been produced in rats by adding vitamin D to a caries-producing diet.

Although both teeth and bones are highly calcified tissues, metabolic effects in the one do not necessarily parallel those in the other. For example, although the percentage of ash of the incisor teeth of rats fed a low-calcium, low-phosphorus diet was lower than that of animals on a normal diet, the absolute amount of ash on the deficient diet increased about 15 per cent during a period of nine weeks. In the normal animals there was an increase of 77 per cent. But during this period there was a reduction of 16 per cent in the absolute amount of ash in the femurs of the animals on the deficient diet, while in the normal animals there was an increase of 38 per cent. Thus there was a continuous deposition of minerals in the incisors of the rats on the deficient diet, while bone underwent demineralization. Furthermore, although the percentage of ash, calcium, and phosphorus of the tibiae of rats may be reduced to about the same extent on a high-calcium, low-phosphorus rachitic diet, and on a low-calcium, high-phosphorus rachitic diet, the values for these constituents are unaffected in the incisors of the rats on the high-calcium diet, but are somewhat reduced on the low-calcium diet. These facts indicate that in some fundamental way there is a marked difference between the mineral metabolism of teeth and bones.

Hormones. Normal tooth growth requires an adequate supply of thyroxine. Hypothyroidism in children causes retardation in dental development although no structural defects of the teeth have been detected in this condition. (Structural defects and impaired calcification have, however, been found after thyroidectomy in monkeys and rabbits.) Reduced activity in the thyroid gland during morphodifferentiation, that is, in utero or in the first year of life, results in a reduction of the size and an alteration in the form of the crowns of the teeth. The size and form of the root may be altered by disturbances in the thyroid gland in later periods. In *hypothyroidism* there is delayed resorption and shedding of the deciduous teeth, whereas in *hyperthyroidism*, which is relatively rare in children, there is early shedding of the deciduous teeth and accelerated dentition.

Deficient parathyroid function may interfere with calcium and phosphorus metabolism to such an extent that growing teeth fail to calcify properly. If this occurs in infancy, enamel hypoplasia and disturbances in the calcification of dentin occur in the tissues calcifying at that time. In experimental hypoparathyroidism in rats, the incisor teeth, which grow throughout life in this animal, become brittle and distorted, whereas fully formed enamel and dentin show no changes. Administration of parathormone to parathyroidectomized rats results in normal calcification of the enamel and dentin calcifying during the period of treatment, but does not restore to normal the structures that were formed in the absence of parathyroid function. In hyperparathyroidism, although calcium and phosphorus are withdrawn from bone, there is no such withdrawal from the teeth. The teeth may become loose as a result of resorption of alveolar bone, but the teeth remain well calcified in spite of the severe drain upon bone.

The pituitary gland plays an important part in the rate of development of the teeth. Dysfunction of this gland usually does not occur before 4 to 6 years of age, that is, before the crowns of the permanent teeth (except the second and third molars) are fully formed. Since eruption of the teeth is retarded, the clinical crown may be smaller than normal. However, the size of the anatomic crown is not affected. Juvenile dental characteristics persist as shown by large pulps and incompletely developed roots. Disturbances occur in the calcification of the dentin and alveolar bone. One of the main dental effects in *hyperpituitarism* is an acceleration in the rate of eruption of the teeth.

In experimental hypogonadism in the male rat, evidence of disturbed calcification was apparent 60 to 90 days after castration. The male hormone therefore appears to have an effect on the calcification of teeth. In hypergonadism in humans, growth, calcification, and eruption of the teeth are accelerated, but not to the same extent as is skeletal growth.

Disturbances of the calcification of growing dentin have been reported in experimental adrenalectomy in the rat.

FLUORINE AND TEETH

The mass occurrence of a characteristic dental defect known as mottled enamel or dental fluorosis was observed about 1908 in the inhabitants of Colorado Springs. Approximately 80 per cent of those who were born and raised in this community were affected to some degree. Those who came to the locality after their teeth had been completely formed were not afflicted with mottled enamel after residence there. Surveys of other communities revealed a similar dental defect. Analysis of the water in the communities where mottled enamel was found revealed the presence of excessive amounts of fluoride. At Bauxite, Arkansas, for example, the fluoride content of the water was 13 to 14 parts per million (13 to 14 mg. per liter), a condition which was subsequently remedied.

Enamel fluorosis is limited to children in whom the enamel of the permanent teeth has not been completed. Enamel formation is completed, except for the third molar, during the seventh to eighth year. It is completed in the third molars between the twelfth and sixteenth years. The disease is characterized by chalky white patches distributed over the enamel, which is pitted and corroded in extreme cases, resulting in loss of the normal translucency. The fluorine content of mottled enamel may be two to three times that of normal enamel; the fluorine content of dentine is likewise increased when water containing excessive amounts of fluoride is ingested during infancy and childhood.

It was found that in communities where dental fluorosis existed there was less dental decay than in comparable communities where the water was practically fluoride-free. In one town where the water supply contained 1.8 parts of fluoride per million parts of water, there was about one-third as much dental decay as in a comparable group of children in another town where the water was almost fluoride-free. About 50 per cent of the children who were raised in the former community showed a mild dental fluorosis. On the basis of this early evidence and subsequent studies, many cities and towns are now adding fluoride to the water supply in an effort to reduce the incidence of dental caries. Fluoride is added to bring the concentration up to approximately 1 part per million, a level at which dental fluorosis is avoided. From the favorable reports regarding reduction of dental caries following this procedure, the addition of fluoride to water to the level of 1 part per million appears to be a worthwhile public health measure.

Another effort to reduce dental decay has involved topical application (application directly to the teeth) of from 0.1 to 2 per cent solutions of sodium or potassium fluoride. According to several reports[20] the incidence of dental caries has been materially reduced by this procedure; other reports have been negative. However, the evidence in favor of topical application of fluoride solutions appears to outweigh that against it. In this connection it is of interest to note that topical application of a 2 per cent sodium fluoride solution to the teeth of dogs has been found to increase the fluoride content of the teeth to a significant degree.[21] Furthermore, enamel which has been treated with fluoride solutions is less soluble in acid than untreated enamel,[22] a fact of importance because in the process of dental decay, the mineral salts of enamel, and later of the dentin, are dissolved by acids which form as a result of the action of enzymes on carbohydrate.

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[20] Knudson and Armstrong: *Am. J. Public Health*, **34**, 239 (1944); Klinkenberg and Bibby: *J. Dental Research*, **29**, 4 (1950).

EXPERIMENTS ON TEETH

Analysis of Teeth. **Place a tooth in 25 ml. of dilute nitric acid and allow to stand over night. On the solution obtained run an analysis for inorganic elements according to the procedure given for bone ash (p. 254). Separate analyses may be made on dentin and enamel. Note the very slight amount of organic matter in enamel.**

Quantitative Analysis of Teeth. **Weigh a clean dry tooth on an analytical balance. Put in a beaker, add 25 ml. of dilute HCl, and let stand over night. Dilute to exactly 100 ml. and filter.**

Determination of Calcium. **Pipet 10-ml. portions of tooth solution into each of two 250-ml. beakers. Add to each 20 ml. of 2.5 per cent oxalic acid solution, 2 drops of methyl red (saturated alcoholic solution), about 70 ml. of distilled water, and then concentrated NH_4OH drop by drop with vigorous stirring. When a precipitate of calcium oxalate begins to form add more ammonia very slowly until the color changes to an intermediate color between red and yellow. Let stand over night. Complete the determination according to directions in Chapter 31. Calculate the percentage of Ca in the tooth.**

Determination of Phosphorus. **Pipet 10 ml. of tooth solution into a 150-ml. beaker, add 2 drops of phenolphthalein solution and NaOH until the color just turns faint pink. Carry out a uranium acetate titration or determine colorimetrically according to the directions given in Chapter 31. Calculate the percentage of P.**

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[21] Hord and Ellis: *J. Dental Research,* **30,** 360 (1951).

[22] Suess and Fosdick: *J. Dental Research,* **30,** 177 (1951).

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10

Muscular Tissue

COMPOSITION OF MUSCLE

Muscular tissue constitutes about 40 per cent of the body weight, and is, therefore, the largest single tissue component of the body. There is great variability in the physiological and finer morphological characteristics of individual muscles, but it is usual to classify them as striated (skeletal, voluntary), cardiac, and smooth muscles. Chemically, striated muscle has been investigated more extensively than cardiac and, especially, smooth muscle.

In round numbers, striated muscle consists of 75 per cent water, 20 per cent proteins, and 5 per cent other solids, such as inorganic salts and the so-called "extractives."

EXTRACTIVES OF MUSCLE

Under the name *extractives* are classed a number of muscle constituents which occur in small amounts in the tissue and may be extracted by water, alcohol, or ether. There are two classes of these extractives, the *non-nitrogenous* and the *nitrogenous*. Grouped under the non-nitrogenous compounds are glycogen, hexosephosphates, lactic acid, inositol, and fat. In the class of nitrogenous extractives are creatine phosphate, purine bases (xanthine, hypoxanthine), uric acid, adenylic acid, inosinic acid, carnosine (ignotine), anserine, and carnitine (novaine). All of these substances, and many more, have been obtained from dead muscle; there is evidence that some of them are produced largely by post-mortem reactions and are not present as such in significant amounts in living muscle. Other extractives besides those enumerated above have been described, and there are undoubtedly still others whose presence remains undetermined. A detailed consideration would, however, be unprofitable in this place.

NON-NITROGENOUS EXTRACTIVES

Lipides. Like most organs, muscles contain storage fat (not dissimilar to that in other depots) as well as essential lipides. Phospholipides predominate among the latter. It has been estimated that mammalian muscles contain on the average 4.5 per cent phospholipides and 0.25 per cent cholesterol, in terms of dry weight. For the heart, these proportions are 7.5 and 0.5 per cent, and for smooth muscle 3 and 0.75 per cent, respectively (Bloor).

Glycogen. The chief carbohydrate of muscle is the polysaccharide glycogen (see p. 87). It may be prepared from muscle or other tissue by extraction with boiling water followed by precipitation with alcohol. By such methods, opalescent solutions of amorphous glycogen are obtained, but by more careful procedures it can be shown that in the living tissue, glycogen occurs in the form of submicroscopic granules, comparable perhaps to vegetable starch grains, although much smaller.

The glycogen content of muscle varies and is reduced by intense activity, but is not, as is liver glycogen, significantly depleted as a result of a demand for carbohydrate elsewhere in the body. Glycogen occurs also in the heart; its behavior in this organ is not the same as in skeletal muscle, and requires further study.

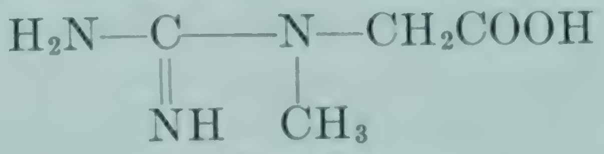
Hexosephosphates. Of the several existing hexosephosphates, resting muscle contains only the so-called Embden ester. This is an equilibrium mixture of glucose-6-phosphate and fructose-6-phosphate (see p. 273), the only intermediates of the glycolytic cycle which accumulate to an appreciable extent under normal conditions.

Inositol occurs in muscle as it does in many tissues, but its mode of combination and its function are unknown (see Chapter 35).

Lactic Acid. This substance occurs in muscle in variable quantity, dependent on the physiological state of the organ. Resting muscle, after careful extraction, contains only very small amounts of lactic acid (e.g., 0.015 per cent in frog muscle) but it is found in larger amount after stimulation, in anaerobiosis, and post mortem. Rigor mortis is associated with lactic acid production and with depletion of adenosinetriphosphate.

NITROGENOUS EXTRACTIVES

Creatine. Creatine, methylguanidinoacetic acid, is found in varying



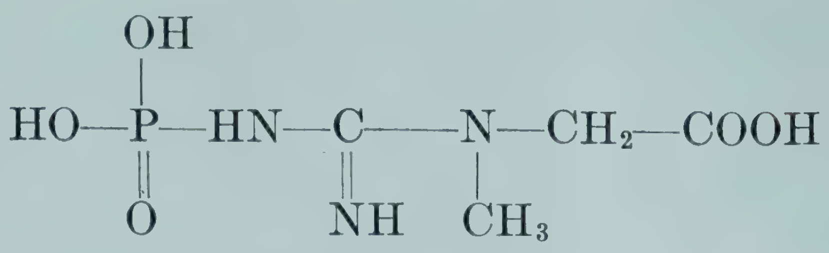
$$H_2N-\underset{\underset{NH}{\|}}{C}-\!\!-\underset{\underset{CH_3}{|}}{N}-CH_2COOH$$

Creatine

amounts in muscles of vertebrates, frequently representing about 0.3 to 0.5 per cent of the wet tissue. The creatine of the body is maintained by synthesis of glycocyamine and its methylation, and a regular excretion of creatine takes place in the form of its anhydride, creatinine, in the urine, dependent somehow on the development of the muscular tissue. In muscular dystrophies, whether spontaneous as in man, or induced in animals by vitamin E deficiency, this creatine excretion is greatly enhanced.

Creatine forms colorless monoclinic prisms which are tasteless to some individuals but bitter to others, and which decompose with marked effervescence at about 291° C. (corr.). It is soluble in warm water and practically insoluble in alcohol and ether. Upon boiling a solution of creatine with dilute hydrochloric acid, it loses water and its anhydride, creatinine, is formed.

Phosphocreatine. Though creatine is present in meat extracts and may also be found in muscle after considerable activity, most of it does not exist as such in perfectly relaxed muscle, but occurs here as phosphocreatine, frequently about 2 millimoles per 100 g. The discovery of



$$HO-\underset{\underset{O}{\|}}{\overset{\overset{OH}{|}}{P}}-HN-\underset{\underset{NH}{\|}}{C}-\underset{\underset{CH_3}{|}}{N}-CH_2-COOH$$

Phosphocreatine

phosphocreatine was preceded by the observation that part of the apparent inorganic phosphate in muscle was in reality present as a labile compound, called phosphagen, which was hydrolyzed by molybdic acid under the conditions of the phosphate determination. This phosphagen was then identified as phosphocreatine by Fiske and SubbaRow. In invertebrate animals, other phosphagens occur; among these, phosphoarginine has been shown to be of widespread occurrence.

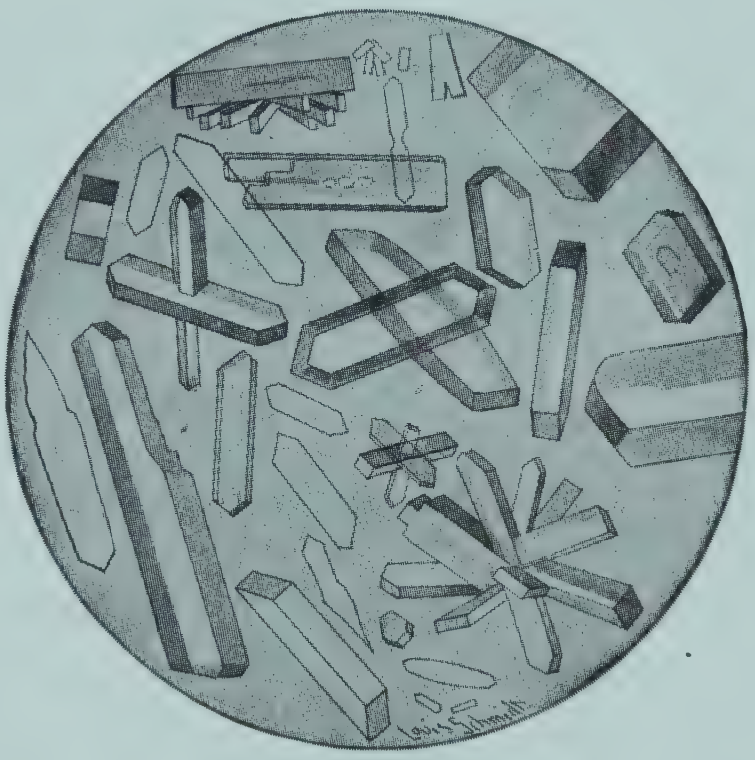


FIG. 73. CREATINE.

Purine Bases and Nucleotides.[1] The purine bases occurring in muscle are adenine, guanine, xanthine, and hypoxanthine (for formulas see Chapter 7). These may exist in the form of nucleic acids and of mono- and oligonucleotides and their breakdown products. Among these, we shall discuss only the adenosine polyphosphates.

In all cases, the basic structure is adenosine, adenine-9-riboside; D(−)ribose being the carbohydrate moiety. This is esterified with phosphoric acid at the 5 position of the ribofuranose, to form adenosine-5-phosphoric acid, also known as adenosinemonophosphate (AMP) or as muscle- or myoadenylic acid. Inosinic acid is a commonly occurring breakdown product of AMP, formed by deamination in muscle extract. Though the nature of adenylic deaminase has not been exhaustively investigated, it appears that myosin, even after considerable purification, displays such enzymic activity.

By attaching further phosphoric acid residues in pyrophosphate linkage, *adenosinediphosphate* (ADP) and *adenosinetriphosphate* (ATP) are obtained. In resting muscle as well as in the mammalian heart, ATP is

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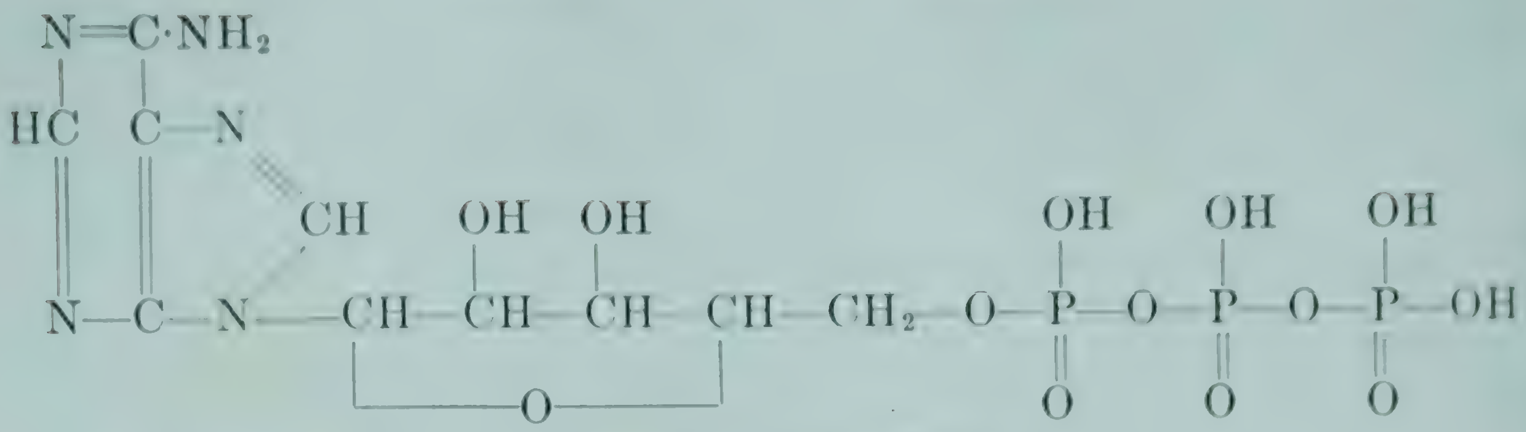
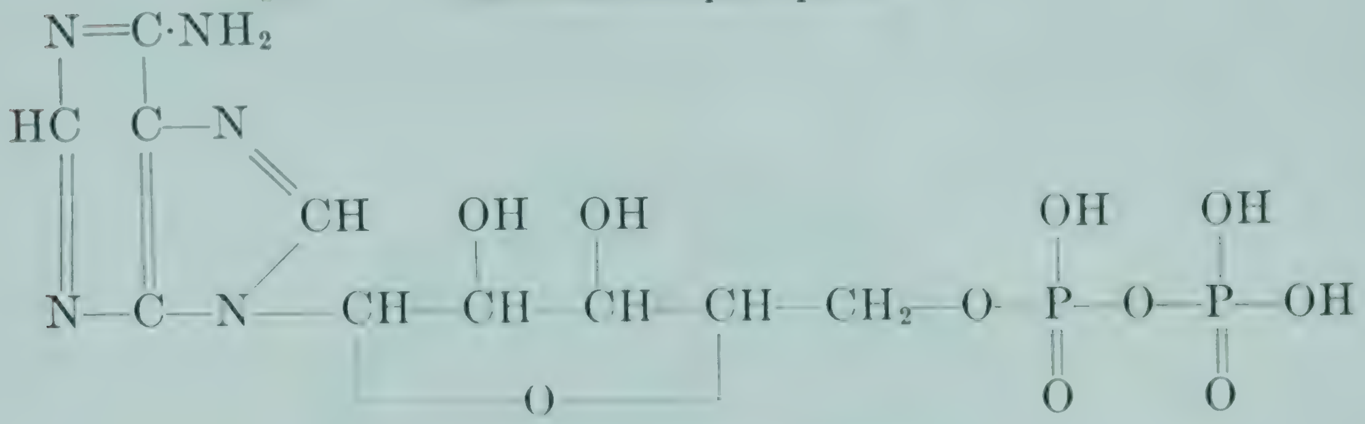
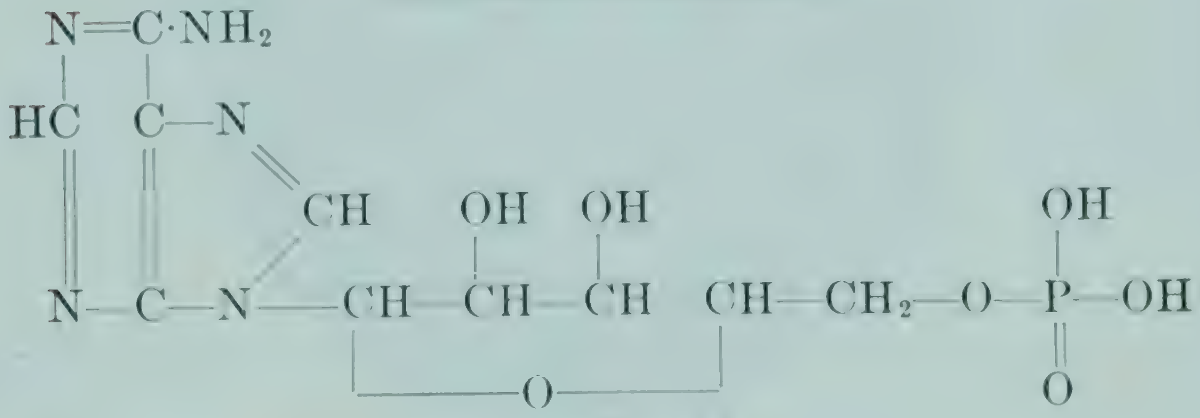
[1] Kanner: *Fortschr. Chem. org. Naturstoffe*, **8**, 96 (1951).



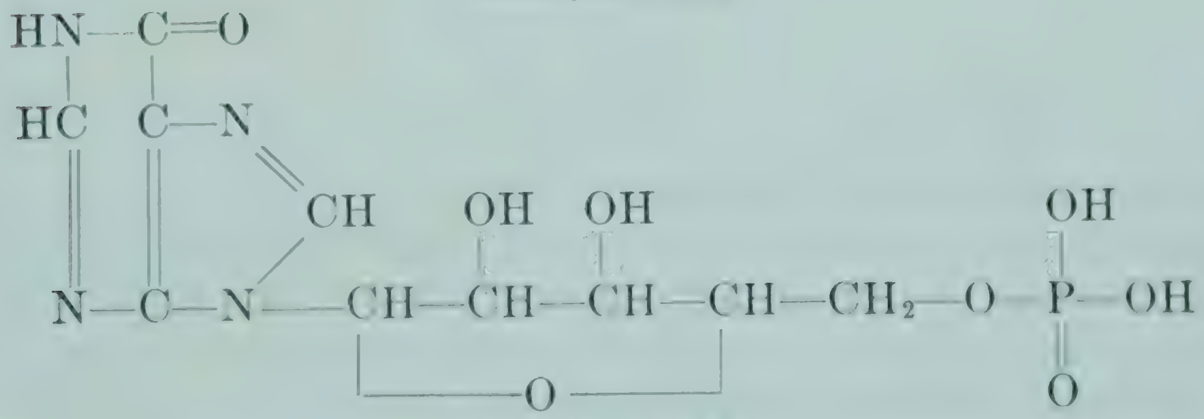
Adenosinetriphosphate



Adenosinediphosphate



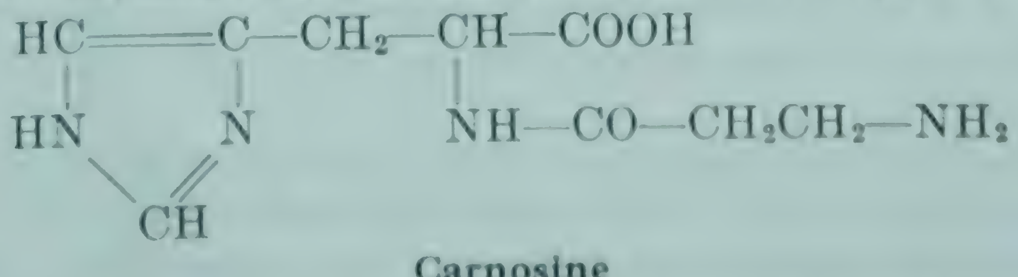
Adenylic acid



Inosinic acid

the predominant form of the adenosine polyphosphates, usually in a concentration of about 0.5 millimole per 100 g.

Carnosine. This substance is a dipeptide of histidine and the amino acid β-alanine, which occurs also in pantothenic acid (see p. 35). Carnosine, often accompanied by anserine (methylcarnosine), is of widespread



Carnosine

occurrence in vertebrate muscle, but its function is not known.[2]

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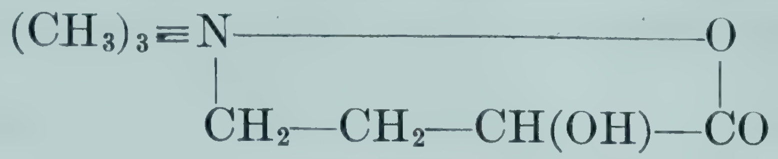
[2] du Vigneaud and Behrens: *Ergebn. Physiol.*, 41, 917 (1939).

Carnitine is a betaine which gives trimethylamine on hydrolysis. Its function in muscle is not known.



Carnitine

Glutamine. Skeletal and, particularly, cardiac muscle contain this substance in higher concentration than other mammalian tissues,[3] about 0.1 per cent. Its function is not known, but reference may be made to the discovery by Krebs[4] that glutamine is essential for the maintenance of the intracellular potassium in brain. (For formula of glutamine, see p. 145.)

INORGANIC SALTS

The predominating inorganic cation of muscle is potassium, occurring in a concentration of about 0.11 molar. It is accompanied by sodium, which, like the chloride ion, is mostly present in the tissue spaces rather than intracellularly. Also significant are calcium and magnesium (about 0.003 and 0.01 molar), which act as activators or inhibitors for many of the enzymes present. These positive ions act in part as counterions for the negatively charged proteins, and are, for the remainder, paired with various phosphoric esters and other anions. Free phosphate occurs, but is easily overestimated due to the lability of phosphocreatine. The actual estimates differ for different muscles, but the results of Hastings for rat muscle may be taken as representative for mammalian muscle.[5]

APPROXIMATE COMPOSITION OF 1 KG. OF MAMMALIAN MUSCLE

	Grams	*Milliequivalents*		*Distribution, per cent*	
		Cations	*Anions*	*Extracellular*	*Intracellular*
Water	760			20	80
Protein	220		~50	10	90
Na		21		80	20
K		107		1	99
Ca		3		>90	<10
Mg		22		1	99
Cl^-			12	>99	<1
HCO_3^-			10	40	60
Phosphates			~80	<1	>99
Total		153	~152		

PROTEINS OF MUSCLE

The protein composition of muscle must be discussed in relation to the microscopic structure of this tissue. We can divide the proteins into those that are constituents of the fibrils and those that occur in the sarcoplasm, even if in practice it may not always be possible to make this distinction with certainty.

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[3] Archibald: *Chem. Revs.*, **37**, 162 (1945).

[4] Terner, Eggleston, and Krebs: *Biochem. J.*, **37**, 139 (1950).

[5] Hastings, in Najjar: *A Symposium on the Clinical and Biochemical Aspects of Carbohydrate Utilization in Health and Disease*, Baltimore, The Johns Hopkins Press. 1952

Stroma. About 20 per cent of the muscle protein cannot be dissolved with any known solvent short of strong urea or alkali solution. This fraction is commonly called stroma, and may consist, in part, of constituents of cell nuclei and connective tissue, including perhaps the sarcolemnar sheaths.

Proteins of the Sarcoplasm. If a muscle is perfused to remove blood, and is then minced and subjected to high pressure, a press juice is obtained which contains about one-fifth of the protein of the minced muscle. This press juice is probably derived from the sarcoplasm. Its main proteins were formerly called myogen and globulin X (Weber); the latter protein has not received further attention. *Myogen* is now recognized as a collective name for a number of water-soluble proteins, and it is probable that this fraction is made up entirely of various enzymes.[6]

In more complete yield, the proteins of the myogen group can be obtained by extracting minced muscle with water or dilute salt solutions (e.g., 0.15 molar KCl). It is then found that about 30 per cent of the muscle protein is contained in this fraction. Such extracts have been analyzed with the aid of electrophoresis, and this method too has shown the complexity of the so-called myogen (Dubuisson).

Myoglobin. A water-soluble protein which deserves separate mention is myoglobin. This protein is similar to hemoglobin (notwithstanding differences in molecular weight (17,000) and other properties) in that it is a heme protein which combines reversibly with oxygen. Its affinity for O_2 is higher than that of hemoglobin, so that the intracellular pigment may be fully oxygenated at O_2 tensions which cause unloading of the blood pigment. The distribution of myoglobin between white and red muscle has most interesting features correlated with physiological differences between the muscles. It may be said, in general, that myoglobin acts as a store of oxygen, although the functional meaning of this is not always clear.

Myoglobin can be released from muscles after crushing injury; because of the small size of the molecule, it is filterable through the renal glomeruli, and appears in the urine.

Proteins of the Fibrils. The two previous categories constitute nearly half of the muscle protein; the remaining half will now be discussed in detail. This fraction, which corresponds to the protein constituents of the fibrils, can be extracted with concentrated salt solutions (e.g., 0.5 M KCl), and has been extensively investigated.

Myosin. When relaxed skeletal muscle is minced and immediately extracted with 0.5 M KCl for an arbitrary length of time at neutral or weakly alkaline reaction, it is found that the extract, in addition to the soluble sarcoplasm proteins, contains a protein, called *myosin*, which can be precipitated by five fold to ten fold dilution of the extract, and can be redissolved in 0.5 M KCl. Myosin solutions thus obtained are distinguished by a high viscosity and *flow birefringence,*[7] but these properties are found to be variable and are largely determined by the duration of the extraction; prolonged extraction gives rise to a *myosin* with higher viscosity and more intense birefringence of flow.[8]

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[6] Herbert, Gordon, Subrahmanyan, and Green: *Biochem. J.*, 34, 1108 (1940).

Actin. Further analysis has shown that these phenomena are due to the combination of myosin with a new protein, *actin,*[9] to form the complex, *actomyosin.* This combination takes place in later stages of the extraction as soon as the adenosinetriphosphate in the system is decomposed; but the release of actin is also promoted by very fine mincing. Pure, actin-free myosin has been prepared in crystalline form by Szent-Györgyi. Its viscosity is moderate, and it shows no flow birefringence when tested with simple methods of observation. Upon combination with actin, however, the viscosity increases greatly, and flow birefringence is pronounced.

Actin can occur in two forms, called globular (G) and fibrous (F) actin. The former can be obtained by extracting acetone-dried muscle powder with water. After addition of salt, the G–F transformation takes place, as evidenced by an enormous increase in viscosity and the development of a strong flow birefringence.[9] These phenomena are ascribed to a suppression of molecular charges by the salt, whereupon the individual molecules unite to form long molecular strands.[10] Only F-actin forms an actomyosin with the described characteristics.

Both actin and myosin are now available as apparently pure proteins. For myosin, purity is achieved by carefully controlled precipitation procedures. The molecular weight of myosin has been difficult to determine, owing to anomalies in its behavior, but is now found to be 600,000, the molecule apparently being a rod 1500 Å long.[11] Actin has been purified by fractional ultracentrifugation of F-actin, followed by careful depolymerization to G-actin; the molecular weight is about 57,000.[12]

Much remains to be elucidated about the exact state in which these proteins occur in the muscle fibril. Electron microscopy has shown, however, that long filaments, presumably actomyosin, run along the fibrils, and that other substances are spaced in a regular fashion, corresponding to the microscopically visible cross striations and other structural details.[13]

Tropomyosin. This protein was crystallized by Bailey after extraction from alcohol-dried muscle. Its behavior is opposite to that of actin in that it is aggregated in the absence of salt, but has its flow birefringence abolished by salt. However, it is not known to be related to contractile phenomena.

There is a good deal of similarity in amino acid composition between myosin, tropomyosin, and actin. Apparently, these proteins are built on another pattern than seed, egg, or blood proteins.

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[7] von Muralt and Edsall: *J. Biol. Chem.*, **89**, 315, 351 (1935).

[8] Banga and Szent-Györgyi: *Stud. Inst. Med. Chem. Szeged*, **1**, 5 (1942).

[9] Straub: *Stud. Inst. Med. Chem. Szeged*, **2**, 3 (1942); **3**, 23 (1943).

[10] Mommaerts: *J. Biol. Chem.*, **198**, 459 (1952).

[11] Mommaerts and Rupp: *J. Biol. Chem.* (in press).

[12] Mommaerts: *J. Biol. Chem.*, **198**, 445 (1952).

[13] Rozsa, Szent-Györgyi, and Wyckoff: *Biochim. Biophys. Acta*, **3**, 561 (1949); Draper and Hodge: *Austr. J. Exp. Biol. Med. Sc.*, **27**, 465 (1949).

BIOCHEMISTRY OF MUSCULAR ACTIVITY

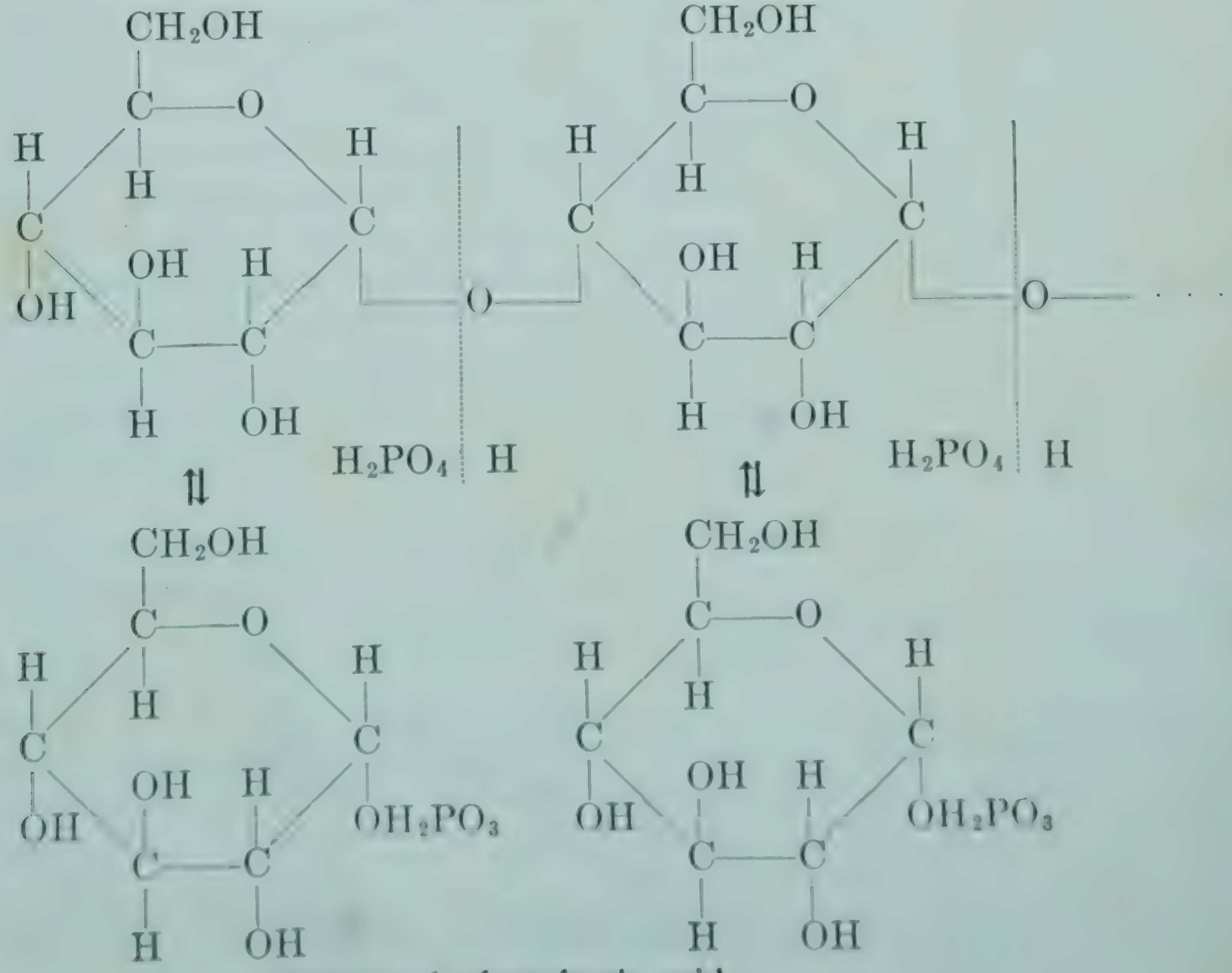
Our present knowledge of the chemistry of muscular contraction is based upon the intensive studies of many investigators over a long period of time. Perhaps no other single phase of animal biochemistry has so persistently attracted the attention of so many biochemists and physiologists. Muscles from both warm- and cold-blooded animals have been studied; skeletal, cardiac, and (to a lesser extent) smooth muscles have been used; and studies have been carried out on the isolated intact muscle, muscle extracts, and enzyme systems obtainable from these extracts. The discussion which follows is a summary of present knowledge, necessarily incomplete and subject to future revision, concerning the chemical processes associated with muscle contraction.

GLYCOLYSIS

Glycolysis is the preponderant metabolic process in muscle under anaerobic conditions, but may also occur aerobically in those muscles which do not contain enough oxidative enzymes to fulfill the metabolic requirements of increased activity. In the human body, considerable amounts of lactic acid appear in strenuous exercise; much of this lactic acid is reconverted to glycogen by the liver.

Glycogenolysis. Breakdown of glycogen can be effected hydrolytically by *amylase*, but this occurs in digestion rather than in tissue metabolism. In muscle and other organs, the breakdown is phosphorolytic instead of hydrolytic, and is catalyzed by the enzyme *phosphorylase*, which has been

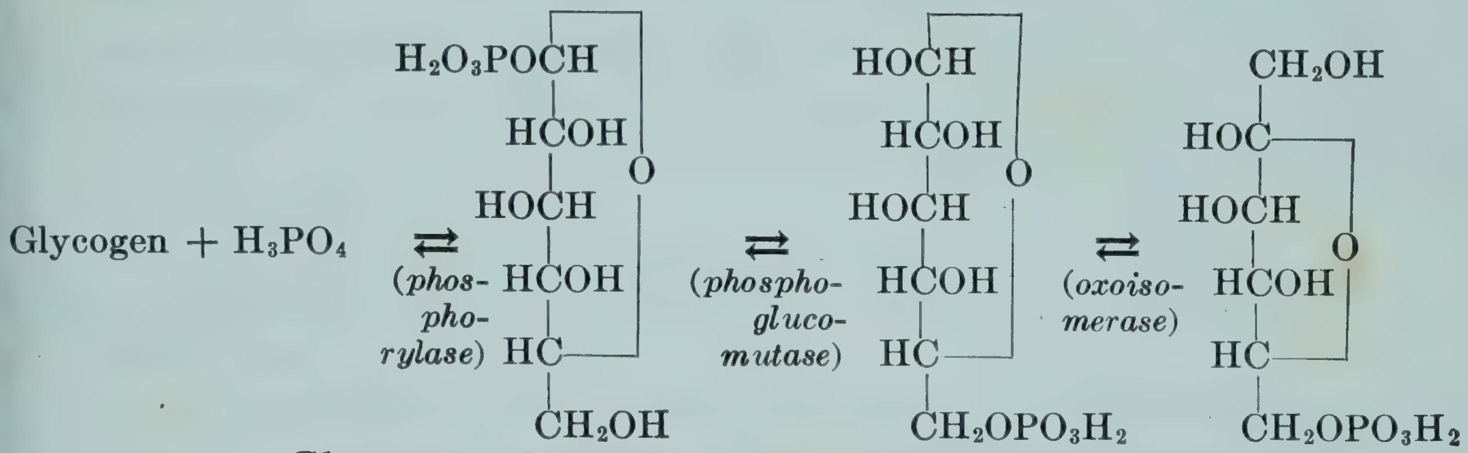
Glycogen



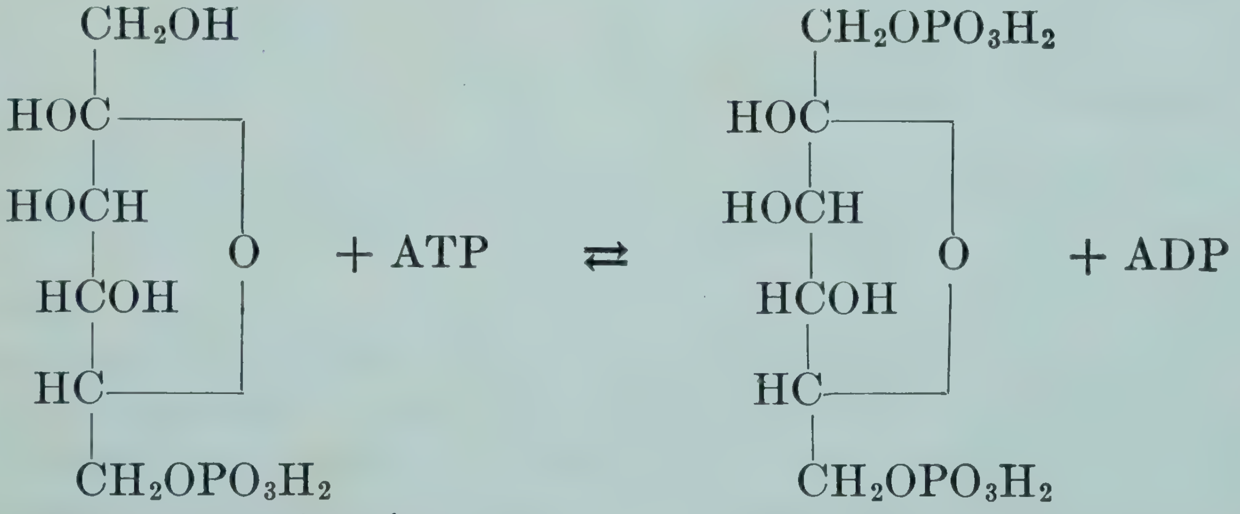
Glucose-1-phosphoric acid

isolated in crystalline form[14] and studied extensively in Cori's laboratory. This reaction may be visualized as shown on p. 272.

This reaction has a number of interesting features. It is an equilibrium reaction which leads to synthesis of glycogen if there is sufficient glucose phosphate relative to phosphate, whereas breakdown occurs in the opposite case. The synthetic reaction requires a small amount of glucose polymer as a primer. In this phosphorylase reaction, an unbranched chain polymer is formed, with all glucose molecules linked by 1,4 linkages; this product is a linear starch, which stains blue with iodine. The synthesis of branched polymers with both 1,4 and 1,6 linkages (of which glycogen is an extreme example) requires the additional participation of a branching factor. This enzyme causes the transfer of parts of straight chains to nonterminal positions. Phosphorylase cannot break 1,6 linkages; these are hydrolyzed by a special enzyme.



Glucose-1-phosphate ("Cori ester") **Glucose-6-phosphate ("Robison ester")** **Fructose-6-phosphate ("Neuberg ester")**



Fructose-6-phosphate ("Neuberg ester") **Fructose-1,6-diphosphate ("Harden-Young ester")**

Phosphorylase is known in two forms, *a* and *b*. The first of these is active as such; but phosphorylase *b* requires the addition of adenylic acid, which seemingly acts as a loosely combined coenzyme. It is probable that phosphorylase *a*, too, contains a nucleotide as its active group, but this is bound strongly, and is not identical with adenylic acid. Various tissues contain an enzyme that converts phosphorylase *a* into *b*; this conversion is observed in muscles after exhaustive activity.

Glucose-1-phosphate is transformed into glucose-6-phosphate by the enzyme *phosphoglucomutase*, crystallized by Najjar; glucose-1,6-diphosphate acts as coenzyme for this reaction. The blood glucose is also a source of glucose-6-phosphate in muscle. The transformation cannot be effected by direct combination of glucose with phosphate, since the equilibrium does not favor synthesis. Instead, glucose is phosphorylated by ATP under the influence of the enzyme *hexokinase:*

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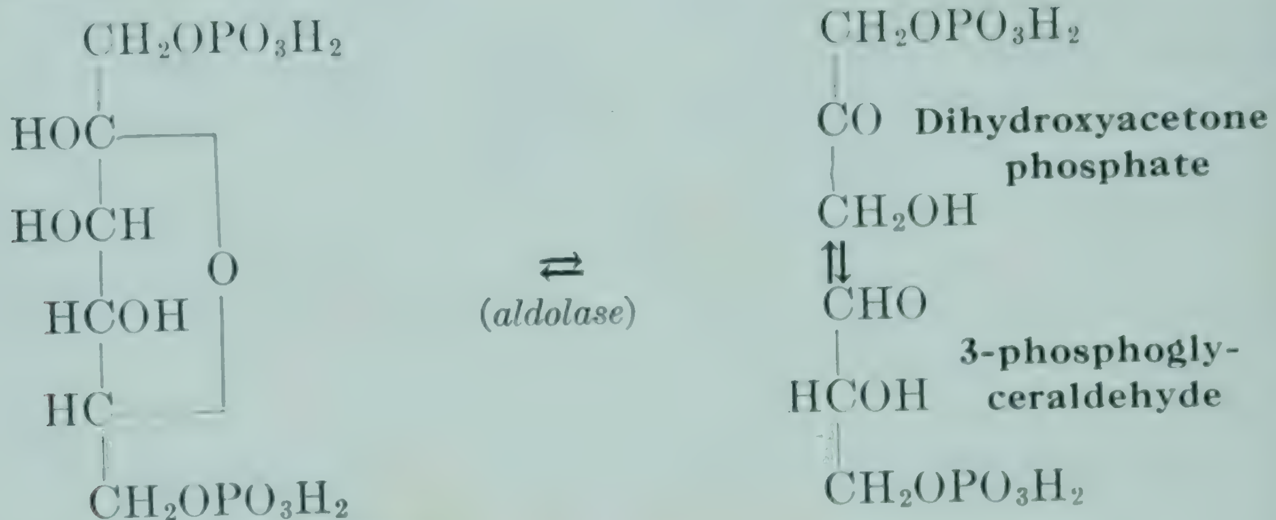
[14] Green, Cori, and Cori: *J. Biol. Chem.*, **142**, 447 (1942).

$$\text{ATP} + \text{glucose} \rightarrow \text{glucose-6-phosphate} + \text{ADP}$$

Formation of Triosephosphate. Glucose-6-phosphate, whether formed from glycogen or glucose, is transformed into fructose-6-phosphate in the presence of the enzyme *oxoisomerase*, and the reaction product is phosphorylated once more by ATP to form fructose-1,6-diphosphate. These steps are shown on p. 273.

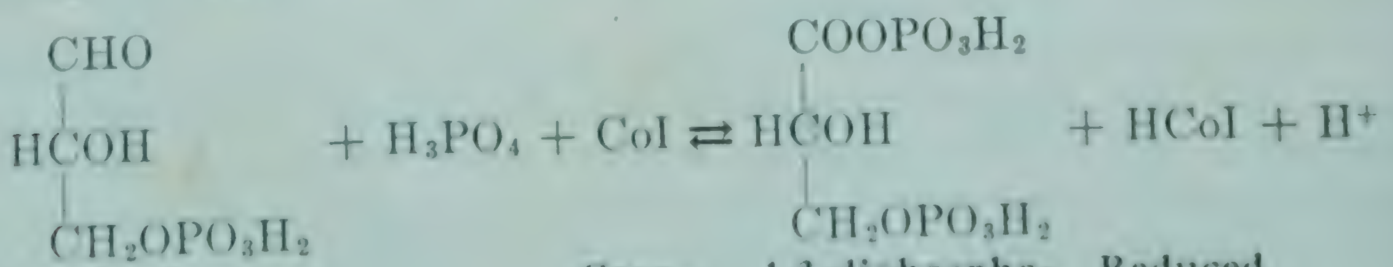
Fructose-1,6-diphosphate is then split under the influence of the enzyme *aldolase* into two isomeric triosephosphates, dihydroxyacetonephosphate and 3-phosphoglyceraldehyde:



Fructose-1,6-diphosphate

These triosephosphates are interconvertible through the action of another isomerizing enzyme. It is the 3-phosphoglyceraldehyde which undergoes further reaction.

Oxidative Step. Triosephosphate is oxidized by pyridine nucleotide (DPN, coenzyme I) and the enzyme *phosphoglyceraldehyde dehydrogenase*, in a reaction which would be expected to yield 3-phosphoglyceric acid. Instead, it is found that 1,3-diphosphoglycerate is formed, while inorganic phosphate (without which the reaction cannot proceed) is taken up (Warburg). One may consider that the dehydrogenation of aldehyde, normally involving a preliminary hydration, in the present case utilizes phosphoric acid instead of water to that effect. The actual reaction mechanism is a problem of considerable interest.[15]



$$\begin{matrix}\text{CHO}\\ |\\ \text{HCOH}\\ |\\ \text{CH}_2\text{OPO}_3\text{H}_2\end{matrix} + \text{H}_3\text{PO}_4 + \text{CoI} \rightleftarrows \begin{matrix}\text{COOPO}_3\text{H}_2\\ |\\ \text{HCOH}\\ |\\ \text{CH}_2\text{OPO}_3\text{H}_2\end{matrix} + \text{HCoI} + \text{H}^+$$

3-phosphoglyceraldehyde **Coenzyme I** **1,3-diphosphoglyceric acid** **Reduced coenzyme I**

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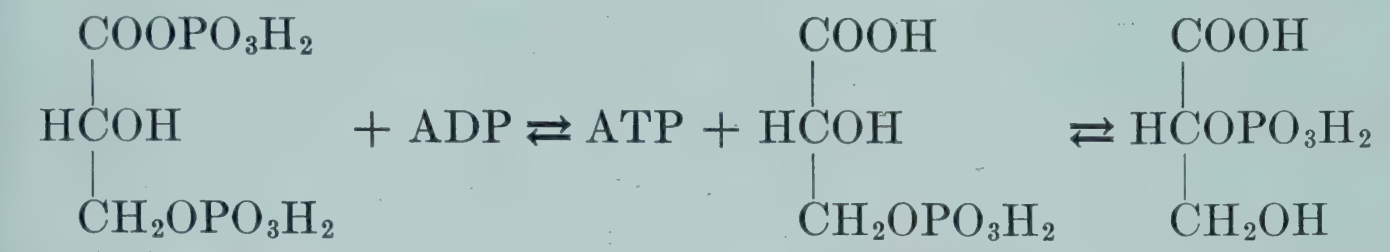
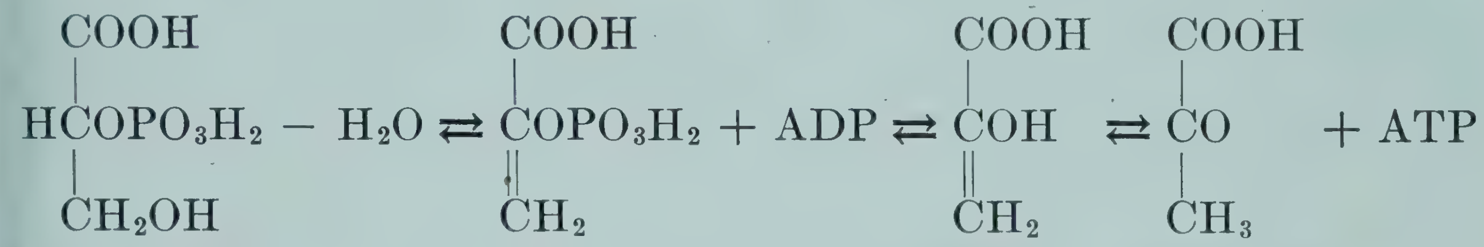
[15] It is the triosephosphate dehydrogenase which seems to be the point of action of iodoacetate poisoning. Under the influence of this drug, glycolysis can be inhibited without immediate effect upon the contractile activity of the tissue (Lundsgaard).

Formation of Pyruvate. Diphosphoglyceric acid is transformed into 3-phosphoglyceric acid, ADP acting as the phosphate acceptor. The product is converted into 2-phosphoglycerate and then, by the enzyme *enolase*, into enol-phosphopyruvic acid. This finally donates its phosphate radical to ADP, yielding pyruvate.



$$\begin{array}{c}COOPO_3H_2\\ |\\ HCOH\\ |\\ CH_2OPO_3H_2\end{array} + ADP \rightleftarrows ATP + \begin{array}{c}COOH\\ |\\ HCOH\\ |\\ CH_2OPO_3H_2\end{array} \rightleftarrows \begin{array}{c}COOH\\ |\\ HCOPO_3H_2\\ |\\ CH_2OH\end{array}$$

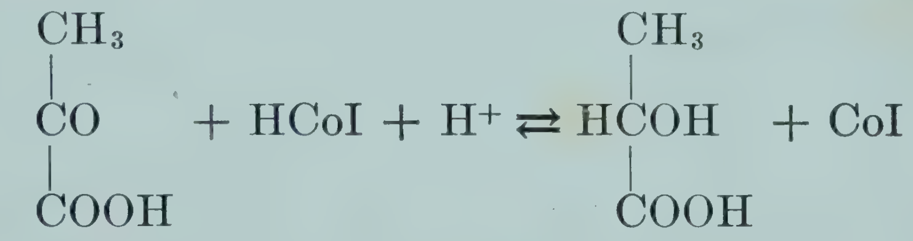
1,3-diphosphoglyceric acid **3-phosphoglyceric acid** **2-phosphoglyceric acid**



$$\begin{array}{c}COOH\\ |\\ HCOPO_3H_2\\ |\\ CH_2OH\end{array} - H_2O \rightleftarrows \begin{array}{c}COOH\\ |\\ COPO_3H_2\\ \|\\ CH_2\end{array} + ADP \rightleftarrows \begin{array}{c}COOH\\ |\\ COH\\ \|\\ CH_2\end{array} \rightleftarrows \begin{array}{c}COOH\\ |\\ CO\\ |\\ CH_3\end{array} + ATP$$

2-phosphoglyceric acid **Enol-phosphopyruvic acid** **Enol form** **Keto form**
Pyruvic acid

Formation of Lactate. In the formation of diphosphoglycerate from triosephosphate, a molecule of coenzyme I had been reduced. This is now reoxidized by pyruvate, reducing the latter to lactic acid, which is the end product of glycolysis.



$$\begin{array}{c}CH_3\\ |\\ CO\\ |\\ COOH\end{array} + HCoI + H^+ \rightleftarrows \begin{array}{c}CH_3\\ |\\ HCOH\\ |\\ COOH\end{array} + CoI$$

Pyruvic acid **Lactic acid**

RESPIRATION

Under aerobic conditions, essentially the same set of reactions takes place as in glycolysis, up to the stage of pyruvate formation. However, the reoxidation of coenzyme I reduced in the triosephosphate dehydrogenation is undertaken by oxidative enzymes and eventually by oxygen. Pyruvate then is not reduced to lactate. Instead, it is completely oxidized in the citric acid cycle (see Chapter 33).

The respiratory enzymes, like cytochromes and cytochrome oxidase (see Chapter 12) are present in different muscles in varying amounts, although muscular activity eventually depends upon respiratory metabolism. There are types of muscle, like the myocardium,[16] which are continuously active and must therefore steadily respire at a sufficiently high rate to meet the energy demands. Such muscles must contain sufficient respiratory enzymes to permit this high rate of metabolism. Other types of muscle, whose activity alternates with periods of rest, frequently are less well endowed with oxidative systems. When such muscles become active, glycolysis takes place in addition to respiration, in an effort to fulfill the energy requirements.

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[16] Much of the classical work on cytochromes, cytochrome oxidase, yellow enzymes and other oxidation factors was done on preparations of mammalian and pigeon heart muscle.

ENERGY-RICH PHOSPHATES

Neither oxygen nor lactic acid is directly involved in contractility, since muscles can contract anaerobically when glycolysis is inhibited with iodoacetate. Furthermore, increased oxygen consumption and lactic acid production occur only after contraction (Embden). Phosphocreatine and ATP are found to be more closely related to the contractile mechanism. These compounds are examples of the so-called high-energy phosphates,[17] compounds which liberate energy when dephosphorylated, as manifested by the large amount of heat produced in their hydrolysis (about 12,000 calories per mole hydrolyzed). This applies to phosphocreatine and to each of the two pyrophosphate linkages in ATP, but not to the ribose-phosphate linkage. The free-energy effect of the splitting is likewise high, so that such compounds can act as phosphate-group donors for the synthesis of other substances, and can, presumably, also serve to yield energy to be transformed into mechanical work.

Generation and Utilization of ATP. The description of the reaction sequence of glycolysis has given examples of how, in the formation of glucose-6-phosphate from glucose and of fructose-1,6-diphosphate from fructose-6-phosphate, ATP acts as a phosphate group donor, and how in other instances, reaction products like diphosphoglycerate or phosphopyruvate transfer phosphate groups to ADP. It is, indeed, the purpose of metabolism to subject the metabolites to such transformations that the phosphate groups contained in them acquire a high group potential, so that they can be transferred to ADP (or AMP, although this, in general, seems to be less important). By such reactions, ATP is regenerated whenever it has been split in other reactions. In glycolysis, 4 moles of ATP are thus formed per mole of hexose decomposed, 2 moles having initially been expended to phosphorylate glucose to the hexose diphosphate stage (only 1 mole in the case of glycogen breakdown; but the formation of glycogen from blood sugar also expends 1 mole of ATP). In respiratory metabolism, there are many other reactions in which ATP is produced, not all of which are known in detail. Aerobic phosphorylation may lead to about 6 moles of ATP generated per mole of O_2 consumed.

Enzymes and ATP. A number of enzymes are known which are of interest in connection with the transformations of ATP. The following enumeration is not complete, but is restricted to some enzymes occurring in muscle. Hydrolysis of ATP is effected by *adenosinetriphosphatases*, or ATPases, which split the terminal pyrophosphate linkage, yielding inorganic phosphate and ADP. At least two such enzymes occur in muscle: one of these is myosin itself (Engelhardt and Ljubimova); the other is a particulate entity similar to the microsomal or mitochondrial fractions isolated from other organs (Kielley and Meyerhof). Both enzymes display a complicated dependence on the ion composition of the medium. The particulate ATPase is activated by magnesium and inhibited by calcium; but for pure myosin the opposite is true. In actomyosin systems the situation is more complicated and has not yet been fully explored. The function of the particulate enzyme is probably not related to contractility. Myosin, on the other hand, is a part of the contractile machinery; hence its ATPase activity is a matter of obvious interest. However, it has been stated that the activity of myosin is not sufficient to account for the actual rate of breakdown occurring in sustained tetanic activity. It is probable that the ATPase activity of myosin is but a reflection of a more fundamental reaction which has not yet been recognized, and which may be related to the occurrence of myosin in an organized state in conjunction with other active materials.

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[17] Lipmann: *Advances in Enzymol.*, 1, 99 (1941).

Phosphocreatine, on the other hand, is not directly hydrolyzed by muscle enzymes. It can transfer its phosphate group to ADP (and also to adenylic acid, AMP) in the Lohmann reaction:

$$\text{ph}\sim\text{creatine} + \text{ADP} \rightleftarrows \text{ATP} + \text{creatine}$$

so that dephosphorylation of phosphocreatine can be effected by a system containing creatine-ATP-phosphopherase, ATPase, and adenine nucleotide. It is held that in living muscle, phosphocreatine acts as a reservoir of ~ph, from which ATP can be restored on short notice. A more fundamental role of phosphocreatine is, however, not at all excluded, and it may be specifically involved in relaxation (Dubuisson).

ATPase removes only one phosphate from ATP, but an enzyme exists (*myokinase*) which catalyzes the transmutation: $2\ \text{ADP} \rightleftarrows \text{ATP} + \text{AMP}$. In combination with ATPase, this enzyme can thus effect the complete dephosphorylation of ATP to AMP, which, as mentioned previously, can then be deaminated to inosinic acid (IMP). The latter is formed after prolonged muscular activity, but it is doubtful whether such far-going breakdown occurs normally under optimal conditions. Formation of IMP may be the normal pathway of autolytic nucleotide catabolism in skeletal muscle, but not in heart or other tissues, where adenylic acid is dephosphorylated rather than deaminated (Kerr).

Other enzymes effect the transfer of phosphate from ATP onto other substrates; thus, *hexokinase* catalyzes the reaction: $\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{glucose-6-phosphate}$. The possible connection of such enzymes, instead of ATPases, with activity metabolism has not yet received sufficient consideration.

True and Apparent ATPase Activity. When an isolated enzyme, myosin or some other, splits ATP into ADP and phosphoric acid, without demonstrable participation of other substances, it is no doubt correct to call such an enzyme "adenosinetriphosphatase." It is an interesting question whether this involves phosphorylation and dephosphorylation of the enzyme protein as intermediate steps, but this would not affect the terminology. It is also possible that in more complex systems a set of reactions would occur as follows:

$$\text{ATP} + \text{X} \rightarrow \text{ADP} + \text{X-ph}$$
$$\text{X-ph} \rightarrow \text{X} + \text{phosphate}$$

In such a case, X would be a coenzyme of the ATPase.

It should be understood, however, that if a splitting of ATP is observed in a crude tissue extract or in an intact organ, this splitting is not necessarily due to an ATPase, with or without coenzyme, but may be linked with other and irreversible metabolic reactions. Thus, the formation of urea in liver from proper ammonia donors through the functioning of the ornithine-citrulline-arginine cycle (see Chapter 33) involves the splitting of ATP. Likewise, formation of glucose-6-phosphate from ATP and glucose, followed by breakdown of the glucose phosphate or its conversion products, would appear as a hydrolysis of ATP. Such additional metabolic components have been referred to as cosubstrates.[18] The breakdown of ATP in complex tissue systems may well be due to such reactions rather than to ATPase.

ATP Breakdown and Muscular Activity. It was shown by Lundsgaard that, when respiration and glycolysis are eliminated, muscular activity is linked with the dephosphorylation of phosphocreatine. As explained, this proceeds presumably via ATP breakdown. The primary breakdown of ATP was demonstrated recently by comparative analyses of relaxed and contracted muscle, both striated and cardiac.[19] It appears that in each case one cycle of activity is linked with the decomposition of maximally 5×10^{-7} moles of ATP per gram of muscle, as predicted on the basis of physiological data. Thus, we regard the dephosphorylation of ATP as the metabolic reaction primarily connected with contraction. The study of heat production in a single twitch (Hill) supports this same sequence: the contraction heat corresponds to splitting of ATP; relaxation (restoring ATP from phosphocreatine) is almost thermoneutral; and the restitution heat depends on the prevalent type of metabolism, whether glycolytic or respiratory. In the more physiological case of tetanic contraction, the various phases overlap.

Although ATP breakdown has been thus correlated with contraction, nothing warrants the assumption that this breakdown is to be ascribed to direct ATPase activity; rather, it may involve complicated cosubstrate reactions or mechanochemical mechanisms.

MOLECULAR MECHANISMS

In muscular contraction, chemical energy is directly transformed into mechanical work, and this transformation takes place in the muscle fibril. An explanation for this phenomenon could be given if it were known just what the molecular structure of the fibril is, how this structure is modified to result in contraction, and how this modification is connected with the breakdown of ATP. This objective has not been reached, much less the corresponding analysis of relaxation and of neuromuscular activation. A number of interesting advances, however, warrant the hope that an explanation of contraction in terms of actomyosin-ATP interaction may eventually be achieved.

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[18] Mommaerts: *Muscular Contraction* pp. 94–95 (see Bibliography). An interesting model of an apparent ATPase system would be formed by choline acetylase, coenzyme A, acetate, choline, and acetylcholine esterase. It is not suggested, however, that this system actually operates as the link between excitation and contraction. See this chapter, p. 279, concerning the possibility that actin may be the cosubstrate.

[19] Mommaerts and Rupp: *Nature*, **168**, 957 (1951); Khairallah and Mommaerts: *Circulation Res.*, **1**, 460 (1953).

It is possible to prepare threads of actomyosin by extrusion of a solution of this protein into a dilute salt solution. Such threads contract when ATP is added, especially in the presence of some magnesium.[20] Oriented and stronger threads can be made by various devices; these thicken during contraction and can lift weights. Finally, one can prepare bundles of actomyosin filaments in their original configuration by extracting all other constituents from muscle strips. Such fiber preparations become thicker when shortening and develop about the same tension as the original muscle would.[21]

Imperfect though the extruded actomyosin fiber is, it permits the study of the individual protein components. Thus, it has been found that neither myosin nor actin alone can form contractile fibers, but that only the combination of myosin with F-actin is capable of contraction. Apart from the cation requirements, it can be said, then, that contractility is the result of the effect of ATP upon an oriented structure of actin and myosin. The mechanism of this interaction is unknown, but the formulation of the problem in these biochemical terms is itself a great advance.

In addition to its role in combination with myosin, actin itself displays some interesting properties. The polymerization of actin from the G to the F state is linked with the breakdown of ATP in a stoichiometric reaction:

$$\text{actin} + \text{ATP} \rightarrow (\text{actin})_{\text{polym.}} + \text{ADP} + \text{phosphate}$$

The amount of actin in one gram of muscle is such that, if it should all polymerize at one time, 5×10^{-7} moles of ATP would be split, which is exactly equal to the amount of ATP broken down in one maximal contraction.[22] This equality makes it likely that polymerization takes place early in the activation phase of the contraction, to be followed by contraction of the F-actomyosin formed.

The participation of actomyosin and its components in contraction is further illustrated by the observation that these proteins can be extracted only from relaxed muscle. Contracted muscle yields no actomyosin or myosin; instead there appears a new protein, contractin, albeit in smaller amount (Dubuisson).

EXPERIMENTS ON MUSCULAR TISSUE

SEPARATION OF EXTRACTIVES FROM MUSCLE

1. *Creatine.* Dissolve about 10 g. of a commercial extract of meat[23] in 200 ml. of warm water. (Test for protein by biuret and coagulation tests, pp. 171, 188.) Precipitate the inorganic constituents by neutral lead acetate, being careful not to add an excess of the reagent (30 ml. of a 20 per cent solution is about the right amount). Write the equations for the reactions taking place

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[20] Szent-Györgyi: *Stud. Inst. Med. Chem. Szeged*, **1**, 17 (1942).

[21] Szent-Györgyi: *Biol. Bull.*, **96**, 140 (1949).

[22] Mommaerts: *J. Biol. Chem.*, **198**, 469 (1952).

[23] Commercial meat extracts vary considerably in their creatine content, and may have to be "fortified" for class use.

here. Allow the precipitate to settle, then filter, and remove the excess of lead in the warm filtrate by hydrogen sulfide. Filter while the solution is yet warm, evaporate the clear filtrate to a syrup, and allow it to stand at least 48 hours in a cool place. Crystals of creatine should form at this point. Examine under the microscope (see Fig. 73). Treat the syrup with 25 ml. of 95 per cent ethyl alcohol, stir well with a glass rod to bring all soluble material into solution, and then filter. When the fluid has drained completely, use a further 10-ml. portion of 95 per cent alcohol to aid in transferring the residue in the beaker to the filter paper, and to wash the material on the filter paper. The purine bases have been dissolved and are in the filtrate, whereas the creatine crystals were insoluble in the alcohol and remain on the filter paper. Remove the crystals and bring them into solution in about 10 ml. of hot water. A little animal charcoal may be added to decolorize the solution. Filter and concentrate the filtrate to small volume. Allow the solution to cool and note the separation of colorless crystals of creatine.[24]

Make the following tests on the crystals:

a. MICROSCOPICAL EXAMINATION. Examine some crystals under the microscope and compare the form with those reproduced in Fig. 73.

b. TRANSFORMATION OF CREATINE INTO CREATININE. Dissolve a portion of the crystals in about 10 ml. of water. Divide into two equal portions. To one portion add 5 ml. of 2 N hydrochloric acid. Evaporate this acidified portion carefully over the free flame and finally to dryness on the water bath. The creatine has been changed into creatinine. Take up the residue in about 5 ml. of hot water, cool, and apply the tests for creatinine as given in Chapter 28 to this extract as well as to the original solution. What are your conclusions?

DIACETYL REACTION. To 5 ml. of a dilute creatine solution add an equal volume of saturated sodium carbonate solution and a few drops of a solution of diacetyl. A pink color should develop. This test has been made the basis of a method for the quantitative determination of creatine (see p. 287).

2. *Preparation of Glycogen.*[25] Grind a few fresh oysters in a mortar with sand.[26] Transfer to an evaporating dish, add water, and boil for 20 minutes. At this point the volume of solution should have been reduced by about one-half. Note the opalescence of the solution. At the boiling point, faintly acidify with acetic acid. Why is this acid added? Filter, and divide the filtrate into two parts.

Test one part of the filtrate as follows:

a. IODINE TEST. To 5 ml. of the solution in a test tube add 5 to 10 drops or more of Lugol's iodine solution, at the same time adding a similar amount of iodine to 5 ml. of water in another tube, this serving as a control. What do you observe? Is this similar to the iodine test upon any other compound with which we have had to deal?

b. REDUCTION TESTS. Does the solution reduce Benedict's solution?

c. HYDROLYSIS OF GLYCOGEN. Add 10 drops of concentrated hydrochloric acid to 10 ml. of the solution and boil for 10 minutes. Cool the solution, neutralize with solid sodium carbonate, and test with Benedict's solution. Does it still fail to reduce Benedict's solution? If you find a reduction how can you prove the identity of the reducing substance?

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[24] For the preparation of pure creatine from creatinine, see p. 801.

[25] For a quantitative experiment showing the effect of diet on the glycogen content of liver, see Chapter 33, p. 1071.

[26] Glycogen may be readily obtained from the livers of well-fed rabbits which have been killed by the intraperitoneal injection of 5 ml. of 25 per cent anhydrous $MgSO_4$ per kg. The excised liver is quickly cut into small pieces and dropped into about 150 ml. of boiling water. Continue then as described above.

d. INFLUENCE OF SALIVA. Place 5 ml. of the solution in a test tube, add 5 drops of saliva, and place in the water bath at 40° C. for 10 minutes. Does this now reduce Benedict's solution?

To the second part of the glycogen filtrate add 3 to 4 volumes of 95 per cent alcohol. Allow the glycogen precipitate to settle, decant the supernatant fluid, and filter the remainder. Transfer the glycogen to a watchglass and heat on a water bath to remove the alcohol; then subject it to the following tests:

a. SOLUBILITY. Try its solubility in cold and hot water, in alcohol, and ether.

b. IODINE TEST. Place a small amount of the glycogen in a depression of a test tablet and add 2 to 3 drops of dilute iodine solution. The same wine-red color is observed as in the iodine test upon the glycogen solution.

NUCLEOTIDES OF MUSCLE

1. Preparation of Adenosinetriphosphate from Muscle. Into the peritoneal cavity of a rabbit inject a solution containing 25 per cent anhydrous magnesium sulfate in the proportion of 5 ml. per kilogram of body weight. When the animal is unconscious, slit its throat and bleed it as thoroughly as possible, then remove the skin, and pack the carcass in ice for an hour or so to chill thoroughly. Excise the muscle tissue of the legs and back, and grind in a chilled meat-grinder. (All of the remaining steps should be carried out in a cold room or with thorough chilling of reagents, containers, etc., by ice.) Mix the minced muscle with an equal weight of ice-cold 10 per cent trichloroacetic acid, and after thorough mixing, filter by suction. Extract the residue with an equal weight of 4 per cent trichloroacetic acid and filter as above. Neutralize the combined filtrates to pH 6.8 with 40 per cent sodium hydroxide solution. Add 50 per cent barium acetate solution to complete precipitation, and allow the precipitate to settle. Decant the supernatant, centrifuge down the precipitate, and wash it once with water.

Treat the precipitate with 0.2 N nitric acid, with stirring, to a pH of 3, or to the first blue with Congo red paper. Centrifuge and pour off the supernatant, rejecting the insoluble material. Add to the supernatant 3 to 5 ml. of Lohmann's reagent[27] for every kilogram of muscle used. Allow to stand at 0° for 15 minutes and centrifuge. Suspend the precipitate in water made faintly acid with nitric acid, treat for 1 hour with hydrogen sulfide to decompose the mercury salt, and filter. Aerate the filtrate to remove excess hydrogen sulfide, then neutralize the filtrate to pH 6.8 with dilute sodium hydroxide solution. Add 25 per cent barium acetate solution to complete precipitation, avoiding an excess. Filter off the precipitate by suction and wash it successively with 1 per cent barium acetate solution, 50 per cent alcohol, 75 per cent alcohol, 95 per cent alcohol, and ether. Dry the product in air and store well-stoppered in the cold. The yield is roughly 3 g. of the barium salt per kilogram of muscle used.

This product usually contains some inorganic phosphate; it may be further purified, with a lower yield, by redissolving in cold 0.1 N hydrochloric acid, neutralizing to pH 6.8 as above, and filtering off the precipitated barium ATP, which is then washed with alcohol and ether and dried as above. The pure salt has the empirical formula $C_{10}H_{12}O_{13}N_5P_3Ba_2 + xH_2O$, the water content depending upon the extent of drying. Purity is usually established by the following criteria: (1) The molecular ratio of nitrogen to phosphorus, as established by total N and total P analyses, should be 5:3; (2) the inorganic phosphate content should be very low; (3) the "labile phosphate" (see below) should be two-thirds of the total phosphate after correcting the latter for any inorganic phosphate present.

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[27] To prepare Lohmann's reagent, add 25 ml. of concentrated nitric acid to 100 g. of mercuric nitrate octahydrate, followed by 25 ml. of water. Warm to dissolve.

2. *Conversion of Barium ATP to Sodium ATP.* Dissolve 200 mg. of the barium salt in 6 ml. of ice-cold 0.1 N hydrochloric acid in a centrifuge tube. Add 60 mg. of anhydrous sodium sulfate dissolved in a little water, stir, and centrifuge. Decant the supernatant and wash the precipitated barium sulfate several times with small portions of cold water containing a trace of hydrochloric acid. Neutralize the combined supernatant and washings to pH 7.5–8.5 as desired, analyze an aliquot for inorganic and labile phosphate, and on the basis of this analysis dilute to the desired concentration of sodium ATP. This solution is relatively stable for some weeks in the cold, but undergoes slow hydrolysis, the extent of which should be established before use.

3. *Conversion of Ba-ATP into Soluble Salts by Means of a Cation-exchange Resin.* Dissolve 200 mg. of the barium salt in 6 ml. cold 0.1 N hydrochloric acid, and dilute to about one-half of the volume eventually desired. Prepare a column of 3 cm. height, in a tube of about 1 cm. width (closed near the bottom with a plug of glass wool or a fritted glass disc), of a cation-exchange resin in the potassium or sodium cycle.[28] Filter the solution through the column at a rate of a few ml. per minute, and wash the column with water. The emerging solution is devoid of barium but is usually still slightly acid. It is adjusted to the desired pH with KOH or NaOH and brought to the required volume.

This procedure is more convenient than the precipitation of barium as sulfate, and is also effective in removing traces of heavy metals which would interfere in many enzyme experiments.[29]

4. *Analysis of Adenosinetriphosphate.* Prepare a solution of adenosinetriphosphate in cold 0.1 N hydrochloric acid, containing approximately 1 mg. of the salt per ml. Analyze aliquots of this solution as follows: (1) INORGANIC PHOSPHATE.[30] Dilute 1 ml. to 4 ml. with water, add 1 ml. of 5 N sulfuric acid, followed by 1 ml. of 2.5 per cent ammonium molybdate and 0.4 ml. of the Fiske-SubbaRow aminonaphtholsulfonic acid reagent (see Chapter 23). Dilute to 10 ml. with water, mix, allow to stand 10 minutes, and read in a photometer at 660 mμ wavelength. Compare with a standard containing 0.05 mg. P, treated in the same way. (2) "LABILE PHOSPHATE." Dilute 0.5 ml. of solution to 4 ml. with water, add 1 ml. of 5 N sulfuric acid, place in boiling water for 30 minutes.[31] Cool, make up to 5 ml. again, and continue with the addition of ammonium molybdate, etc., as described above. The phosphate

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[28] Made by cleaning Amberlite IR 100 or 120, or Dowex 50, by alternate washing in acid and base, finally bringing the resin to the required state with about 0.5 M Na_2CO_3 or K_2CO_3, followed by rinsing with water.

[29] Good preparations of K- or Na-ATP are now available commercially at moderate prices.

[30] In this and subsequent analyses, if barium is present, a precipitate of barium sulfate will form during the procedure. This is centrifuged down after color development and before reading.

[31] According to Lohmann, the "labile phosphate" of ATP is liberated by 7 minutes' hydrolysis in N hydrochloric acid at 100° C. Fiske and others claim that 15 minutes are required. When N sulfuric acid is used, a 30-minute period has been found necessary.

content, corrected for the inorganic phosphate already present, represents the "labile phosphate" of ATP. It should be two-thirds of the total ATP phosphorus.[32] (3) TOTAL PHOSPHORUS. Transfer a 1 ml. portion of the ATP solution to a large test tube or micro-Kjeldahl flask and add 2.5 ml. of 5 N sulfuric acid. Heat over a microburner until the water has boiled off and the residual fluid is brown or black. Add 1 drop of 30 per cent hydrogen peroxide and boil until clear. Cool, transfer to a 25-ml. graduated flask, with washings to about 18 ml. volume. Add 2.5 ml. of ammonium molybdate and 1 ml. of aminonaphtholsulfonic acid reagent. Dilute to 25 ml., mix, and allow to stand 10 minutes. Compare in a photometer against a standard containing 0.1 mg. P digested with acid, etc., just as was the unknown. A blank of water alone should also be run through the entire procedure, to correct for any phosphorus in the reagents. The total P of the sample, corrected for any inorganic P present, represents ATP phosphorus. (4) TOTAL NITROGEN. Determine by any suitable micro-Kjeldahl method (see Chapters 23 and 31). A 4-ml. portion of the ATP solution described above will contain about 0.3 mg. of nitrogen. The N:P ratio on a molecular basis should be 5:3. (5) ULTRAVIOLET ABSORPTION. Measure the absorption spectrum of a suitably diluted neutral or acid solution of ATP (containing about 10 to 40 mg. per liter) between 230 and 300 mμ. The absorption maximum should be situated at 258 to 260 mμ. The adenine content can be calculated on the basis of an extinction coefficient of 1.6 at the maximum for a 10^{-4} molar solution. The adenine:N:P ratio should be 1:5:3, on a molecular or atomic basis.

5. *Preparation of Adenylic Acid from Adenosinetriphosphate* (*Kerr*). Dissolve 2.5 millimoles of barium adenosinetriphosphate (93 mg. of ATP phosphorus equals one millimole) in a flask containing about 100 ml. of 0.1 N hydrochloric acid, and add a few drops of phenolphthalein followed by sufficient barium hydroxide solution to produce a permanent pink color. Attach a reflux condenser to the flask and heat to boiling over a free flame. Boil for 30 minutes, maintaining the reaction just alkaline to phenolphthalein by the continuous addition of barium hydroxide solution in small portions through the condenser tube. Cool to room temperature, add sufficient N hydrochloric acid to the flask contents to dissolve the precipitate, and dilute to about 1,750 ml. with water. Again neutralize to phenolphthalein with barium hydroxide, and allow the precipitate to settle. Remove the supernatant by decantation and filtration, discarding the precipitate. To the supernatant add sufficient acetic acid to make the final acid concentration 0.2 per cent, followed by 50 ml. of 20 per cent mercuric acetate solution per liter of fluid present. Allow the precipitate to settle overnight, separate it by decantation and centrifugation, and wash it once with 0.5 per cent mercuric acetate solution. Suspend the washed precipitate in about 50 ml. of water containing a few drops of 2 N sulfuric acid and treat with hydrogen sulfide for one hour. Filter off the precipitated mercuric sulfide and pass air through the filtrate to remove excess hydrogen sulfide. The volume at this point should not be over 25 ml. per millimole of starting material. Add acetone to the solution at 20° C. to the first permanent turbidity (not over 1.5 volumes). Allow to stand on ice overnight, filter and discard the precipitate. Bring the filtrate to room temperature and again add acetone to the point of turbidity. Again chill overnight. Separate the crystalline adenylic acid by centrifugation. Repeat the addition of acetone as described until a total of 3 volumes has been

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[32] Determination of the acid-labile P (2 moles per mole ATP) is a method frequently used for the determination of ATP. It is a popular misconception that this can also be used to prepare adenylic acid. In reality, the nucleotide is split by this procedure into adenine, ribose-5-phosphate, and inorganic phosphate.

added. Drive off excess acetone from the separated adenylic acid by gentle warming. Recrystallize by dissolving in the minimal amount of hot water, filtering quickly, and allowing the filtrate to stand in the cold overnight. Centrifuge or filter off the crystals and repeat the recrystallization procedure five or six more times to obtain a final product which has the theoretical N:P ratio of 5:1 and the correct melting point of 189° C. The final crystals are washed with a little alcohol and ether and dried. The mother liquors from the various recrystallizations may be saved, combined, and treated with 3 volumes of acetone, followed by recrystallization as above, to obtain more adenylic acid which is less pure.

PREPARATION AND REACTIONS OF MYOSIN AND ACTIN

1. Preparation of a Crude Myosin Solution. Anesthetize and kill a lean rabbit, as described for the preparation of ATP, and chill and excise the musculature in the same manner. Mince the tissue in a chilled meat-grinder, and extract immediately for 20 minutes in the cold with 3 volumes of 0.6 M KCl, 0.05 M $NaHCO_3$. Collect the extract by centrifugation or straining through several layers of gauze, and dilute with 10 to 12 volumes of cold water (redistilled in a glass still, or purified through a mixed-bed ion-exchange resin, in a so-called water-demineralizer which should be made of plastic without metal). Allow the precipitated myosin to settle in the cold, siphon off most of the supernatant solution, and collect the myosin by centrifugation. Redissolve the myosin by the addition of a measured amount of 2 M KCl solution, and add water until the final KCl concentration is 0.5 M.

2. Formation of Actomyosin. Proceed with the extraction of minced muscle as in the previous experiment, but incubate the tissue with the alkaline KCl solution for various lengths of time, up to 18 to 24 hours in the cold, with occasional stirring. Take 50 ml. aliquots at regular intervals, and obtain the extracts from these by centrifugation followed by filtration through glass wool. Formation of actomyosin is indicated by a strong rise in viscosity and flow birefringence (see below). Actomyosin can be prepared from the extract by precipitation and resolution, as described for myosin.

3. Observation of Flow Birefringence. A simple apparatus can be assembled with the aid of two 2-inch polaroid discs mounted vertically above each other about 4 to 6 inches apart and illuminated from below. The polaroids are crossed, so that no light is transmitted. A 30-ml. glass beaker is selected such that, when examined in the polariscope thus assembled, its bottom does not show significant birefringence. A crude or purified solution of actomyosin is placed in the beaker to about 1 cm. depth, and this solution, while being observed between the crossed polaroids, is gently rotated by swirling the beaker by hand. The rotating solution shows a dark cross (the isoclyne cross) on an illuminated background. This phenomenon is due to the orientation of very long particles (molecules or swarms) in the streaming gradient, which leads to a regular alignment giving rise to birefringence, except for those orientations that are parallel with the vibrational axes of the polarizer and analyser.

4. Preparation of Crystalline Myosin.[33] Prepare minced rabbit muscle as previously described, and extract 300 g. in the cold with 1 liter of 0.3 M KCl,

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[33] This procedure combines different features of the methods of Szent-Györgyi, of Weber, and of Mommaerts and Parrish.

0.15 M K-phosphates (pH 6.5) for 15 minutes with gentle stirring. Add 4 liters of cold water, and strain through several layers of gauze. Dilute with water to 12 liters final volume, and leave in the cold for 3 hours. Siphon off the supernatant solution as far as possible, collect the protein by centrifugation, dissolve it by the addition of 60 ml. of 2 M KCl and 30 ml. of a solution 0.25 M with respect to K_2HPO_4 and KH_2PO_4 each, and add water to a final volume of 300 ml. (pH 6.5 to 6.8, ionic strength 0.5). Clarify this solution by centrifugation and filtration through glass wool or Filter-cel. Add 240 ml. of water, and remove the precipitated actomyosin by strong centrifugation. (The solution may remain somewhat turbid through micellar aggregation of myosin at this ionic composition.) Place the solution in a large, cooled container and slowly (10 minutes) add 4 liters of cold water with continuous stirring. The myosin will precipitate in the form of fine, microscopically visible needles which give a silky sheen to the suspension. The steps for the removal of actomyosin and the crystallization may be repeated, and the myosin finally dissolved in 0.5 M KCl, and dialyzed against this solvent.

Pure myosin shows no flow birefringence with the simple method described in Experiment 3 of this section.

5. *Demonstration of Adenosinetriphosphatase Activity of Myosin Preparations.* Place 2 ml. of myosin solution (diluted in 0.5 M potassium chloride so as to contain 1 to 2 mg. of protein) in each of two test tubes. Add 1 ml. of 4 per cent sodium bicarbonate solution and 0.5 ml. of 0.1 per cent anhydrous calcium chloride solution. To Tube 1, which is a control, add 0.5 ml. of 20 per cent trichloroacetic acid solution. To each tube add 0.5 ml. of sodium ATP solution containing a known amount (0.1 to 0.2 mg.) of "labile phosphorus." Dilute the contents of the control tube immediately to 10 ml. with water, mix, and filter. Allow the second tube to stand at room temperature for 30 minutes, then add 0.5 ml. of 20 per cent trichloroacetic acid solution, dilute to 10 ml., mix, and filter. Determine the inorganic phosphate content of each filtrate as described above, using a 2 ml. aliquot. Correct for the value of the control by subtraction. What fraction of the labile phosphate of the ATP has been converted into inorganic phosphate by the myosin? If desired, this experiment may be repeated at varying time intervals to establish the rate of enzyme action.

6. *Preparation of Muscle Fibrils* (*Perry*). Rabbit muscle is dissected, chilled, and minced as for the preparation of myosin. The mince is homogenized in a Waring blendor for 2 minutes with 8 volumes of 0.08 M borate buffer, pH 7.1, at 0° C. The following operations are likewise carried out at this temperature. The homogenate is centrifuged 15 minutes at about 600 g., and the sediment is resuspended in the original volume of borate. This is homogenized once more for 2 minutes. This suspension is centrifuged for 20 minutes at 600 g.; the supernatant solution is discarded, while the lighter-colored upper layer of the sediment is taken up in fresh borate buffer. This suspension is then freed from coarser material by centrifugation for 5 minutes at 300 g. The suspension is then subjected to several cycles of centrifugation (20–30 minutes at 600 g.) and resuspension. Toward the end of the preparation 0.1 M KCl may be used instead of borate buffer.

Such suspensions form turbid viscous fluids which show a strong flow birefringence. Microscopic observation reveals the presence of thin threads, in many of which the cross-striation is clearly visible. The addition of ATP results in rapid contraction, revealed by loss of birefringence and precipitation of flocculent material. Fibril preparations are very active as adenosinetriphosphatase, but contain also myokinase and adenylate deaminase activity; the myokinase can be removed by washing ATP-contracted fibrils with water or dilute KCl solution.

7. *Contractile Fiber Preparation* (*Szent-Györgyi*). A rabbit is killed as for the preparation of myosin, and eviscerated. The body is placed in a refrigerator for 15 minutes and is finally cooled in ice. The psoas muscle is exposed by removing the sides and the anterior part of the body, and is liberated by placing the index finger under it and moving the finger in a longitudinal direction. Then the muscle is lifted slightly and punched through with a probe or with small forceps with closed tips so as to separate a fiber bundle about 2 mm. in diameter from the bulk of the muscle. This fiber bundle is then separated over the whole length by moving the forceps parallel to the fibers. A small stick (applicator rod) is placed alongside, and the fiber bundle is tied to it at both ends; whereupon, it is cut free. Other fiber bundles are isolated in the same fashion. Each bundle with its rod is placed in 50 per cent glycerol at 0° C. (maintained with melting ice in a thermos bottle, placed in a refrigerator). The fluid is exchanged twice for fresh, precooled 50 per cent glycerol, at daily intervals, after which the fibers can be stored at −20° C. for months. Before use, the fibers are placed in 15 per cent glycerol at 0° C. for one hour, and are then split longitudinally into finer threads by pulling with fine forceps.

Contraction can be observed by placing such fibers in fresh muscle extract (made by suspending freshly minced muscle in an equal volume of boiling water and filtering through cloth), or in 0.2 per cent neutralized ATP in 0.1 M KCl, 0.001 M $MgCl_2$.

8. *Preparation of Actin* (*Straub, with modifications of Bailey and Tsao*). Muscle is extracted for 10 minutes, and the mince is diluted threefold with water, as in the preparation of crystalline myosin. After straining through gauze, the residue is washed by gentle stirring in 4 volumes of 0.05 M $NaHCO_3$ (the pH becomes about 7) and then in 10 volumes of water; each washing lasts 20–30 minutes. The residue is pressed as dry as possible in a cloth, and is then extracted twice in a Waring blendor for 1 minute with 2 volumes of cold *n*-butanol (let it stand for 30 minutes), and finally 3 times with 2 volumes of acetone, after which it is dried in the air.

The dry fiber is extracted with 25 volumes of water or (in order to assure a good yield) ATP solution (about 60 mg per liter, pH 8.2). The material does not wet easily, and must be thoroughly stirred for 30 minutes in the cold. The solution is filtered by suction and centrifuged.

This extract contains actin in the globular form (usually about 5 mg. per ml., of about 50 per cent purity), so that its viscosity is low, and no birefringence is shown. After the addition of KCl to a final concentration of 0.1 M, rapid formation of fibrous actin takes place. This is evident from an increase in viscosity, frequently leading to a gelation of the solution, and by the appearance of a most intense flow birefringence.

SOME CHEMICAL DETERMINATIONS ON MUSCLE

In this section a few selected analyses on muscular tissues are given without specification as to the origin of the tissue or its physiological state. These experiments can be performed on frog muscles, rat diaphragms, or other suitable materials.

1. *Extraction Procedure.* About one g. muscle is extracted in the cold with 3 ml. of 0.5 N perchloric acid, and the residue (after filtration or centrifugation) is re-extracted with 2 ml. of the same reagent. The combined extracts are rapidly neutralized to a faint pink color with phenolphthalein, and are left in the cold to assure precipitation of the potassium perchlorate (the residual solubility of which is about 0.05 M at 0° C.). The solution is filtered quantitatively into a 10 ml. volumetric flask, the phenolphthalein is removed by extracting twice with ether (withdrawing the ether with an eyedropper), and the solution is brought to volume. Alternatively, neutralization can be controlled with the glass electrode.

2. *Chromatographic Separations of Adenosinepolyphosphates* (*Cohn and Carter*). A column is prepared (2 cm. height, 1 cm. diameter) of the anion-exchange resin Dowex 1 in the chloride cycle[34] in a suitable chromatography tube. A muscle extract, prepared as described above, is diluted 5 times with water (in order to reduce the salt content, which would counteract retention by the column), the pH is adjusted to 9.5–10, and the solution is passed slowly through the column, which retains the nucleotides quantitatively. Elution is effected by successive application of 100–150-ml. portions of 0.003 N HCl; 0.01 N HCl, 0.02 M NaCl; and 0.01 N HCl, 0.2 M NaCl. These solvents elute AMP and IMP, ADP, and ATP, respectively. The eluates are collected in fractions of about 10 ml., in each of which the nucleotide is determined by measurements of the ultraviolet absorption, as described on p. 283 for ATP or, if no facilities for this are available, by the Bial test, p. 845.

3. *Separation of Adenosinepolyphosphates by Paper Chromatography.* Small amounts of muscle extract (e.g., 0.02 ml.) are applied to strips of suitable filter paper (e.g., Whatman No. 4); the size of each spot is kept small by intermittent application while drying after each delivery in a current of air. The chromatogram is developed by means of a solution of 60 volumes of *n*-propanol, 10 volumes of water, and 30 volumes of concentrated ammonia (Eggleton). Nucleotides can be located by observation in short-wave ultraviolet light by means of a Mineralight lamp. The experimental details of paper chromatography are described on p. 18.

4. *Determination of Free Creatine.* For this and the following determinations, the tissue extraction should be carried out with especial care and rapidity. It is advisable to use 0.5 N perchloric acid which contains 20 per cent ethanol, cooled to −10° C., and to perform all manipulations at the lowest possible temperature until the extract is neutralized. In a 10-ml. volumetric flask, mix 1 ml. extract, a few ml. of water, 2 ml. of alkaline α-naphthol solution (0.5 g. freshly dissolved in 1 liter of stock alkali: 60 g. NaOH and 160 g. Na_2CO_3 per liter), and 1 ml. of diacetyl (a freshly prepared 1:20 dilution of a 1 per cent stock solution). Compare the color or absorbancy (at 520 mμ) with that given by a standard solution containing 0.005 mg. creatine treated in the same manner.

5. *Determination of Free Inorganic Phosphate and of Phosphocreatine* (*Ennor and Stocken's adaptation of the method of Berenblum and Chain*).

(a) INORGANIC PHOSPHATE. In a separatory funnel, place 2 ml. of neutralized muscle extract, 1 ml. of 0.25 N H_2SO_4 containing 4 per cent NaCl, and 1 ml.

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[34] The resin is cleaned by alternate washings with acid and alkali, and is finally treated with 3 N HCl and washed with water until neutral.

of 0.5 per cent ammonium molybdate. Add these reagents in rapid sequence (5 seconds in total being permissible) and shake *immediately* for 10 seconds with 8 ml. of isobutanol. After separation of the phases (which is facilitated by the salt added to the sulfuric acid solution), wash twice with 4 ml. of 1 N H_2SO_4, swirling but not shaking the contents of the funnel each time. In parallel experiments, run a blank (2 ml. water) and a standard (2 ml., containing about 10 μg. P). The isobutanol phases are then shaken for one-half minute with 10–12 ml. of stannous chloride solution (1 ml. of a stock solution of 10 g. $SnCl_2$ in 25 ml. of concentrated HCl, freshly diluted with 200 ml. 2 N H_2SO_4). The aqueous layer is removed, and the alcoholic layer is quantitatively transferred to a 10-ml. volumetric flask, the funnel is washed with ethanol, and the solution brought to volume with this solvent. Colorimetric readings are then performed at about 610 mμ.

(b) INORGANIC PHOSPHATE + CREATINEPHOSPHATE. Place 1 ml. extract in a separatory funnel with 2 ml. 1 N H_2SO_4 and 1 ml. 5 per cent molybdate, and keep this at room temperature for 15 minutes. Run a blank determination with 1 ml. water, and a standard with 1 ml., containing 10 μg. P. After it has stood, extract with isobutanol, wash with H_2SO_4, and reduce with $SnCl_2$ as described for inorganic phosphates.

6. *Demonstration of Phosphates and Magnesium in Muscle* (*Hürthle's Experiment*). Tease a very small piece of frog's muscle on a microscope slide. Expose the slide to ammonia vapor for a few moments, then adjust a cover glass, and examine the muscle fibers under the microscope. Note the large number of crystals of ammonium magnesium phosphate distributed everywhere throughout the muscle fiber, thus demonstrating the abundance of phosphates and magnesium in this type of muscle preparation (see Fig. 226).

7. *"Fuchsin-frog" Experiment*. Inject a saturated aqueous solution of fuchsin S into the lymph spaces of a frog two or three times daily for one or two days, in this way thoroughly saturating the tissues with the dye. Pith the animal (insert a heavy wire or blunt needle through the occipito-atlantoid membrane), remove the skin from both hind legs, and expose the sciatic nerve in one of them. Insert a small wire hook through the jaws of the frog and suspend the animal from an ordinary clamp or iron ring. Pass electrodes under the exposed sciatic nerve, and after tying the other leg to prevent any muscular movement, stimulate the exposed nerve by means of make and break shocks from an induction coil. The stimulated leg responds by pronounced muscular contractions, whereas the tied leg remains inactive. Continue the stimulation until the muscles are fatigued. The muscular activity has caused the production of lactic acid and this in turn has reacted with the injected fuchsin to cause a pink or red color to develop. The muscles of the inactive leg still remain unchanged in color.

The color of the fuchsin S when injected is red; this substance acts as a pH indicator, however, and is colorless at the pH of normal tissues. Upon stimulating the muscles, as explained above, lactic acid is formed and the resultant lowering of pH regenerates the original color of the dye.

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11

Nervous Tissue

In common with the other solid tissues of the body, nervous tissue contains a large amount of water. The percentage of water present depends upon the particular form of nervous tissue, but in all forms it is invariably greater in the gray matter than in the white matter. Embryonic nervous tissues also contain a larger percentage of water than the tissues of the adult. The gray matter of the brain of the fetus, for instance, contains about 92 per cent of water, whereas the gray matter of the brain of the adult contains approximately 83 to 84 per cent. Adult whole brain (mixed gray and white matter) has an average water content of 77 to 78 per cent; human spinal cord contains about 75 per cent of water.

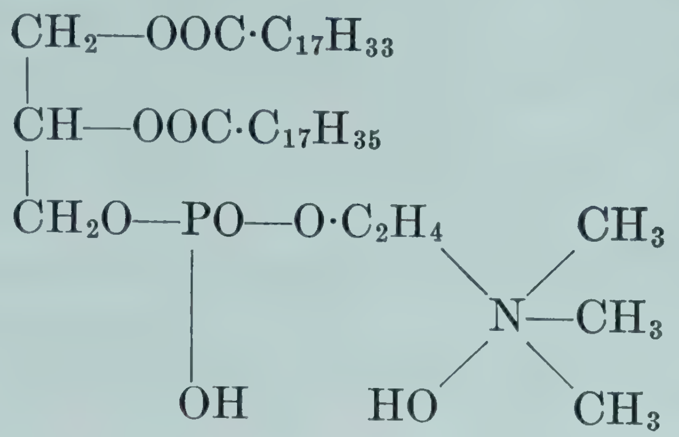
The solids of nervous tissue include proteins, lipides, extractives, and inorganic salts. In adult whole brain the relative amounts of these various components, in percentage of the total solids, are approximately as follows: proteins 38 to 40 per cent, lipides 51 to 54 per cent, and extractives (including inorganic salts) 8 to 9 per cent. Other parts of the nervous system may show a somewhat different distribution.

The Proteins. The proteins of nervous tissue which have been fairly well characterized include several globulins, nucleoprotein, and the albuminoid neurokeratin. Two of the globulins coagulate at 47° C. and 70° to 75° C., respectively; the nucleoprotein coagulates at 56° to 60° C. The relatively low coagulating temperature of one of the globulins of nervous tissue may be of importance in connection with the nervous manifestations of heat prostration.

The Lipides. Many types of lipides occur in nervous tissue. Those which have been reasonably well characterized include (1) phospholipides or phosphatides, (2) cerebrosides or glycolipides, (3) sulfolipides or sulfatides, (4) gangliosides, and (5) cholesterol. Other types will doubtless be recognized as progress continues in this field. It will be noted that the lipides are chiefly of the compound type; there is little or no true fat in nervous tissue. For example, of the 54 per cent of cerebrum solids which are composed of lipide material, the phosphatides constitute approximately 28 per cent, cholesterol 10 per cent, cerebrosides 7 per cent, and the remaining 9 per cent consists chiefly of sulfatides and other relatively poorly characterized and similar material. It has been shown that the relative amounts of certain of the lipides present in the brain may be influenced by age and sex.

PHOSPHOLIPIDES (PHOSPHATIDES). The phospholipides or phosphatides include (1) lecithins, (2) cephalins, and (3) sphingomyelin.

Lecithins. The lecithins are sometimes spoken of as "phosphorized fats." Their relationship to the fats is indicated by the formula of a typical lecithin.



Lecithin

This lecithin is called oleyl-stearyl-lecithin. On hydrolysis it yields oleic acid, stearic acid, glycerol, phosphoric acid, and choline. Chemically, this compound may be regarded as an ester of choline and a phosphatidic acid, and is therefore a phosphatidyl choline. The phosphatidic acids themselves contain fatty acids, glycerol, and phosphoric acid (but no choline), and have been isolated from tissues as the calcium salts. There is some question whether phosphatidic acids exist as such in tissues, or are produced from lecithins by enzymatic removal of choline during the treatment of the tissue.

There are different lecithins, depending upon the character of the fatty acid radicals. Most of the naturally occurring lecithins contain at least one unsaturated fatty acid radical, but there are instances where both fatty acid radicals are found saturated. The saturated fatty acids found include palmitic and stearic. The unsaturated acids are oleic, linoleic, linolenic and arachidonic (see Chapter 3). Representative types of the lecithin molecule have been synthesized in the laboratory, and in some instances found to be identical with material isolated from natural sources.

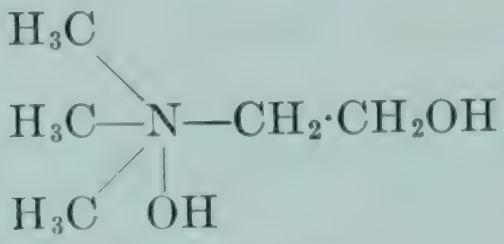
It is clear from the structure given above that two isomeric forms of a lecithin molecule are possible, depending upon whether the phosphoric acid–choline group is attached to the α (terminal) or β (middle) carbon of the glycerol residue; such lecithins are known as α- and β-lecithins respectively. There is no satisfactory evidence at present that β-lecithins occur naturally.

The lecithins are not confined to the nervous tissues but are found in nearly all animal and vegetable tissues, where they appear to be primary constituents of the cell. Lecithin is soluble in chloroform, ether, alcohol, benzene, and carbon disulfide. It may be precipitated from chloroform or alcohol-ether solution by acetone. Lecithin may be caused to crystallize in the form of small plates by cooling the alcoholic solution to a low temperature. It has the power of combining with acids, bases, and certain salts, such as cadmium chloride, and the hydrochloric acid salt forms a double salt with platinic chloride.

Pure lecithin is colorless; it readily turns brown on exposure to air, presumably because of oxidation of the unsaturated fatty acids present. The choline–phosphoric acid portion of the lecithin molecule is highly water-soluble; the fatty acid portion is insoluble in water but soluble in fats.

Lecithin forms colloidal solutions in water which are of the hydrophilic or emulsoid type (see Chapter 1). If a portion of solid lecithin is placed in water and then observed under the microscope, the lecithin will be seen to diffuse out into the water in the form of long curving strands ("myelin forms") which bear a remarkable resemblance to the protoplasmic protuberances of lower forms of life. The hydrophilic nature of the lecithin molecule may be of importance in connection with the structure and properties of cell membranes.

Choline is one of the products of hydrolysis of lecithin, accounting for about 15 per cent of the molecule. It is trimethyl-hydroxyethyl-ammonium hydroxide, and has the following structure:



Choline

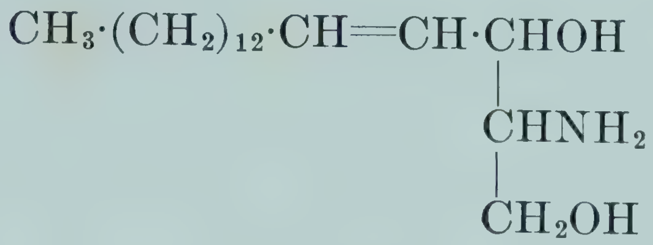
Choline is a moderately strong base, and forms a crystalline double salt with platinic chloride. In addition to its presence in the lecithin molecule, choline is found widely distributed elsewhere in nature, either free or combined, and appears to play an important part in a variety of physiological processes. In combination with acetic acid the compound acetylcholine is of significance in nerve activity, as discussed later. In the diet choline has been shown to be a source of the labile methyl group (methyl attached to nitrogen or sulfur), which is an important dietary requirement. Further aspects of the biochemistry of choline will be found in Chapters 33 and 35.

Cephalins. The cephalins differ from the lecithins in both solubility and chemical structure. Differences in alcohol solubility are used to separate the lecithin fraction from the cephalin fraction of tissue extracts. Chemically, the cephalin fraction is found to be heterogeneous and to contain at least three types of compounds. Two of these types differ from the lecithins in that the choline of the lecithin molecule is replaced by either ethanolamine (colamine), $CH_2OH \cdot CH_2NH_2$, or serine, $CH_2OH \cdot CHNH_2 \cdot COOH$. These two types of cephalins are therefore known as phosphatidyl ethanolamine and phosphatidyl serine respectively. As with the lecithins, the nature and type of fatty acid residues joined to glycerol in the cephalins may vary, and the problems of isomerism are similar to those discussed above for lecithin.

A third type of cephalin, less well defined than the other two types, yields inositol as a characteristic hydrolytic product, along with fatty acids, phosphoric acid, ethanolamine, and possibly galactose and tartaric acid. According to Folch, the inositol-containing phosphatide from brain, called diphosphoinositide, is chemically distinct from the more complex inositol-containing phosphatide isolated from the soybean by Woolley and named lipositol. Further work is required to clarify this situation.

Lysolecithin and lysocephalin are prepared by treating lecithin or cephalin with cobra venom. An enzyme present in the venom splits off the unsaturated fatty acid radical leaving the structure otherwise unchanged. Compounds of this type have a strong hemolytic action on red cells and the hemolytic action of venom is probably brought about in this way. These lysophosphatides combine readily with cholesterol, molecule for molecule, the resulting compound having no hemolytic power.

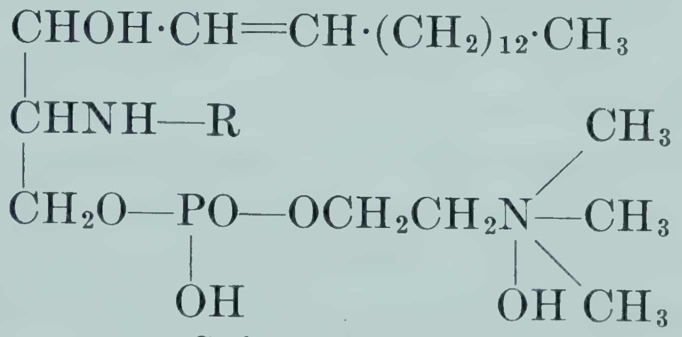
Sphingomyelin. Sphingomyelin represents a third type of phospholipide found in nervous tissue. It is a diaminomonophosphatide made up of a molecule each of fatty acid, phosphoric acid, and the bases choline and sphingosine.



$$\begin{array}{r}CH_3\cdot(CH_2)_{12}\cdot CH{=}CH\cdot CHOH\\ |\quad\;\;\\ CHNH_2\\ |\quad\;\;\\ CH_2OH\end{array}$$

Sphingosine

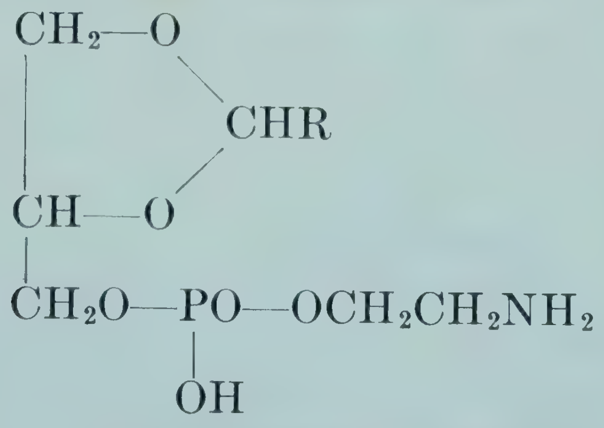
Among the fatty acids found have been the saturated acids—lignoceric, $C_{24}H_{48}O_2$, and another acid, probably hydroxystearic. The fatty acid is apparently in an amide linkage with the amino group of sphingosine. The complete structure would be as follows, R indicating the fatty acid radical.



Sphingomyelin

The content of sphingomyelin (and of lecithin as well) is considerably increased in the liver and spleen in the condition known as Niemann-Pick's disease.

ACETAL PHOSPHOLIPIDES (PLASMALOGENS). Phospholipides which under suitable conditions give a positive test for aldehydes were first described by Feulgen and given the name *plasmalogen.* These substances are found to contain fatty acid aldehydes joined in acetal linkage to the glycerol of compounds having the basic lecithin or cephalin structure.



Acetal phospholipide

In this structure, R represents the remainder of a fatty acid aldehyde chain, and the nitrogenous base is ethanolamine. The crystalline acetal phospholipide isolated by Thannhauser et al.[1] from beef brain contained palmitic and stearic aldehydes, glycerol, phosphoric acid, and aminoethanol. The presence of a serine-containing acetal phospholipide in brain has also been reported. Nothing is known as yet concerning the function or physiological significance of acetal phospholipides.

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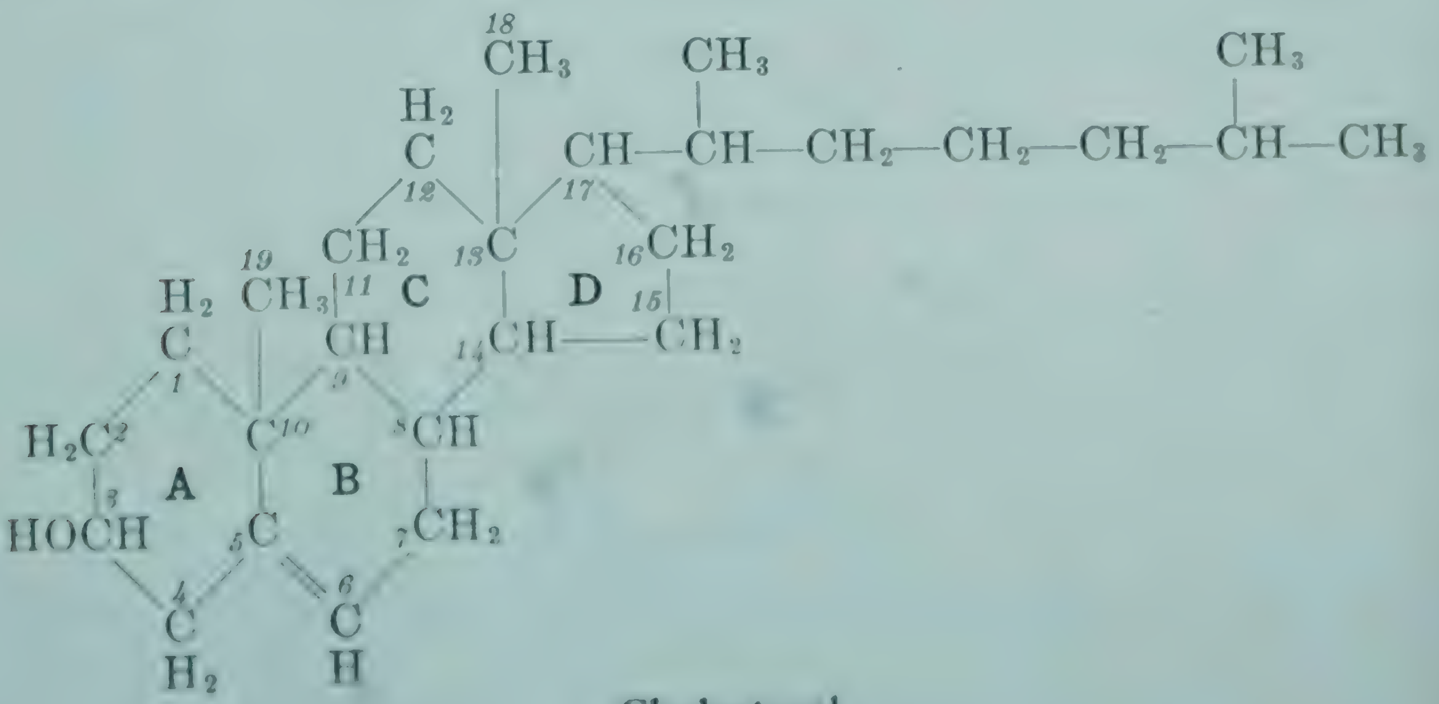
[1] Thannhauser, Boncoddo, and Schmidt: *J. Biol. Chem.* 188, 417 (1951).

CEREBROSIDES. The cerebrosides (sometimes called glycolipides because of their carbohydrate content) are lipides containing carbohydrate, one fatty acid, and sphingosine, but no phosphoric acid or glycerol. Examples include phrenosine, kerasine, nervone, hydroxynervone. Two general categories are now known: (1) galactolipides, containing galactose as the component sugar, and (2) glucolipides, containing glucose. Galactolipides are the more abundant form; glucolipides were originally identified in spleen from Gaucher's disease, a condition in which excessive amounts of cerebrosides accumulate in the spleen, but are now known to be a normally occurring component of various tissues. Cerebrosides appear to be an important constituent of the myelin sheath of medullated nerve fibers, which contain about three times as much cerebroside as is found in nonmedullated nerves. The fatty acid in phrenosine is the hydroxy acid phrenosinic acid $C_{24}H_{48}(OH)COOH$, and in kerasine it is lignoceric acid. The glycolipides are relatively less soluble in ether and more soluble in hot alcohol than the phosphatides.

GANGLIOSIDES. Gangliosides represent a class of cerebrosides first described by Klenk, which are found in brain gray matter, spinal cord, and other tissues. On hydrolysis they yield one molecule each of a base, presumably sphingosine, stearic acid, neuraminic acid, and three molecules of galactose.

SULFOLIPIDES (SULFATIDES). The sulfolipides are similar to the cerebrosides, but characteristically contain sulfuric acid esterified with cerebronic (hydroxylignoceric) acid. Sulfolipides containing sphingosine, galactose, cerebronic acid, sulfuric acid and potassium in equimolecular proportions have been characterized by Blix.

CHOLESTEROL. Cholesterol, one of the primary cell constituents, is present in fairly large amount in nervous tissue. It is a monatomic alcohol containing one double bond, with the empirical formula $C_{27}H_{45}OH$. Its structure is as follows:



Cholesterol

The carbon atoms are numbered as shown for purposes of reference. The four rings A, B, C, and D form the cyclopentano-perhydrophenanthrene nucleus, which is characteristic not only of cholesterol and other plant and animal sterols but also of a wide variety of naturally occurring compounds of surprisingly diverse physiological significance. These compounds include (*a*) the bile salts, (*b*) the steroid hormones, (*c*) the sterol vitamins, (*d*) the aglycone portion of the cardiac glycosides (digitalis, etc.), (*e*) the sapogenins, derived from plant saponins; and one might also include here the carcinogenic hydrocarbons of the phenanthrene type. The possible interrelationship of these varied compounds, whose chemical structures are fundamentally similar, has attracted considerable attention; it has been shown for example that the cholesterol of the bile is a precursor of the chemically similar cholic acid derivatives also present.

Cholesterol is soluble in ether, chloroform, benzene, and hot alcohol. It crystallizes in the form of thin, colorless, transparent plates (see Fig. 101). Cholesterol is present in bile, and occurs abundantly in one form of biliary calculus. It is also present in blood and its quantitative determination is of clinical importance (see p. 580). It has been found in feces, wool fat, egg yolk, and milk, frequently in the form of its esters of higher fatty acids. It is found in the tissues of all animals. The cholesterol present in the animal body has its origin in animal foods or synthesis in the body; work with isotopes has shown that acetic acid may be a major intermediate in this synthesis. It does not appear to arise from plant sterols. That cholesterol may be synthesized in the animal body is proved by experimental work using isotopes as well as by such facts as that hens continue to lay eggs containing cholesterol when fed a cholesterol-free diet and that the tissues of the rat contain the usual cholesterol content when fed a diet containing no sterols.

Various isomers and derivatives of cholesterol are found in plant and animal tissues; only a few may be considered here. A further discussion of steroid isomerism will be found in Chapter 26. *Allocholesterol* or *coprostenol* is an isomer of cholesterol differing only in that the double bond is in the 4-5 instead of the 5-6 position. *Coprosterol* or *coprostanol* is formed by the addition of two hydrogen atoms at the double bond in allocholesterol. It is found in the feces, being formed by bacterial reduction. *Ergosterol* (a provitamin D) differs from cholesterol in having two more double bonds, one in the 7-8 position and one in the side chain, with an additional methyl group in the side chain which thus becomes $—CH(CH_3)\cdot CH{:}CH\cdot CH(CH_3)\cdot CH(CH_3)\cdot CH_3$. On suitable irradiation ergosterol finally yields *calciferol* with a rupture of the B ring. 7-Dehydrocholesterol, found in the skin, is also activated by irradiation to produce a compound with vitamin-D activity. *Stigmasterol* obtained from plant sources differs from ergosterol in having only one double bond in the nucleus (like cholesterol) and in having the second methyl group in the side chain replaced by an ethyl group. The *phytosterols* or plant sterols comprise several different sterols. According to Schoenheimer, the bulk of plant sterols do not appear to be absorbed by the body.

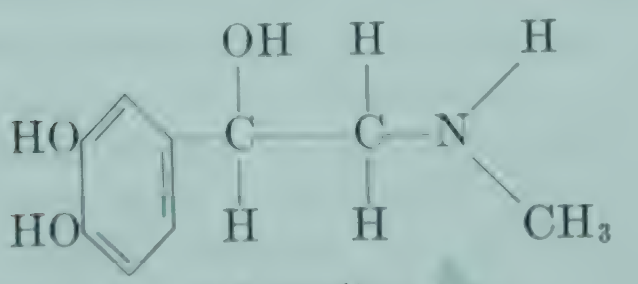
Inorganic Salts. Nervous tissue yields about 1 per cent of ash which is made up in large part of sodium and potassium chlorides and phosphates. The inorganic salts of fresh brain and nerve tissue consist largely of sodium and potassium chloride, potassium being in excess over sodium. The content of sodium and potassium ions varies from one part of the brain to another, and has attracted considerable attention in relation to a possible role in nerve action. The student is referred to Page's book for a further discussion of this subject (see Bibliography).

CHEMICAL CHANGES IN NERVOUS ACTIVITY

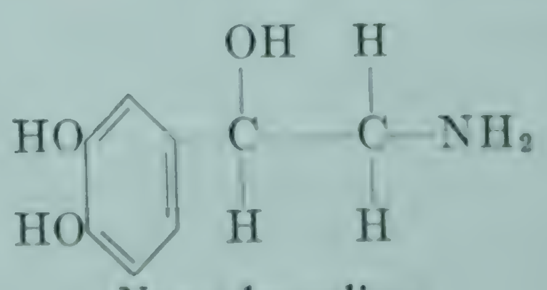
The carbohydrate metabolism of nervous tissue appears to follow much the same course as that of muscle tissue, going by way of pyruvate and lactate anaerobically, and the *citric acid cycle* aerobically. Since glycogen is low in amount in the brain and glucose appears to be the major carbohydrate substrate, this organ is more immediately dependent upon the blood sugar supply. Nerves consume oxygen and liberate heat to a greater extent following stimulation.

The transmission of impulses along a nerve or from nerve fibers to muscle fibers or secretory cells, or from one nerve fiber to another across synapses in ganglia, is thought to involve chemical changes, either directly or as the source of potential differences. According to one view, the nerve action current results from a redistribution of diffusible ions between the center of the nerve and the periphery, taking place successively along the nerve fiber. This redistribution of ions may be under the control of chemical agencies.

At the motor nerve endings in voluntary muscle, stimulation appears to liberate the compound acetylcholine (see below). Involuntary muscles and secretory cells are controlled by two sets of nerves, sympathetic and parasympathetic. The parasympathetic fibers liberate acetylcholine at the nerve endings in such cells. Stimulation of such nerves causes smooth muscle fibers, as in blood vessels, to relax with dilation of the vessels. Stimulation of the sympathetic nerves brings about the liberation of at least two substances whose structures are known; these are adrenaline (epinephrine, suprarenine) and nor-adrenaline(nor-epinephrine, arterenol, demethylated epinephrine).



Adrenaline

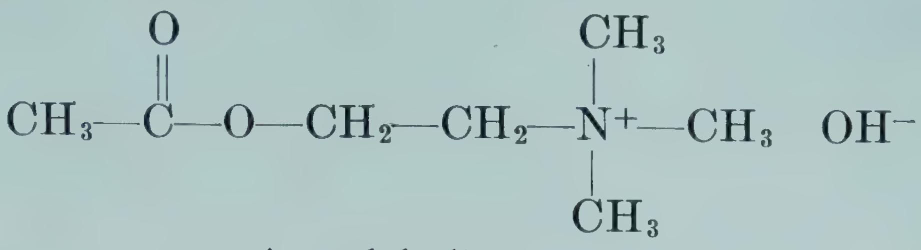


Nor-adrenaline

Usually only one of these two *neurohormones*, as they are called, predominates in a particular organ or extract thereof. There is some basis for the view that nor-adrenaline is a precursor of adrenaline. Of the two possible optical isomers of each compound, the levorotatory form is biologically much more active than is the dextrorotatory form.

Nerves which release adrenaline or nor-adrenaline on stimulation are known as adrenergic nerves; those releasing acetylcholine are called cholinergic nerves. The sympathetic fibers are adrenergic nerves; cholinergic nerves include motor nerves, all preganglionic fibers, and the postganglionic parasympathetic fibers.

Acetylcholine. As stated above, acetylcholine is believed to be the neurohormone of cholinergic nerves. Chemically, acetylcholine is the acetic acid ester of choline:



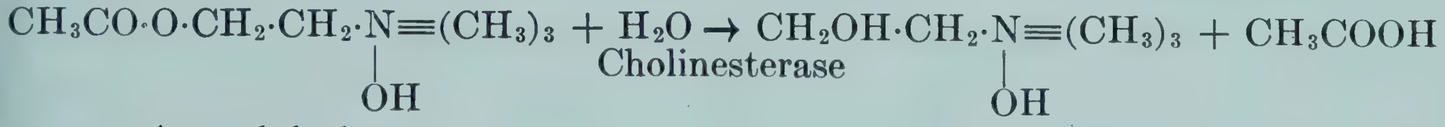
$$CH_3-\overset{\overset{O}{\|}}{C}-O-CH_2-CH_2-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}-CH_3\quad OH^-$$

Acetylcholine

The structure as shown is that of the base; the compound is usually obtained commercially as a halogen salt, the iodide being less hygroscopic than the chloride or bromide. Solutions of acetylcholine salts show slight hydrolysis in neutral solution, which is accelerated in alkaline solution. Free acetylcholine may be detected, usually by bioassay, in low concentration in nervous and other tissues. A relatively much greater proportion of the total detectable acetylcholine content of tissues is in a bound inactive form. The acetylcholine released by nerve stimulation presumably comes from this source; the mechanism is not known.

Brain tissue contains an enzyme, choline acetylase, which promotes the synthesis of acetylcholine from choline and acetate, through the mediation of adenosinetriphosphate (ATP) and coenzyme A. This reaction is similar to other biological acetylations involving coenzyme A (see Chapter 33), choline acting as acceptor of the acetyl group.

Physiological control over the excessive accumulation of acetylcholine constantly being produced by normal nerve impulses appears to be effected by the enzyme cholinesterase, which catalyzes the hydrolysis of acetylcholine into the relatively inert compounds choline and acetic acid.



$$CH_3CO\cdot O\cdot CH_2\cdot CH_2\cdot \underset{\underset{OH}{|}}{N}\equiv(CH_3)_3 + H_2O \xrightarrow[\text{Cholinesterase}]{} CH_2OH\cdot CH_2\cdot \underset{\underset{OH}{|}}{N}\equiv(CH_3)_3 + CH_3COOH$$

Acetylcholine **Choline** **Acetic acid**

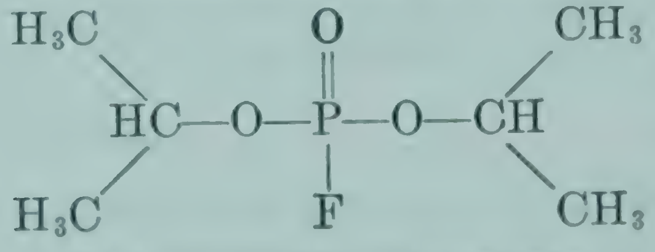
Cholinesterase activity is found in the nervous tissues of all animal species thus far studied; it is also present in mammalian red blood cells, blood plasma, to a certain extent in other tissues, and in snake venom. The cholinesterase activity of the brain varies with location, the caudate nucleus being much more active than other portions of the brain. The enzyme has not been obtained in a pure or crystalline form; the most potent preparations have been made by Nachmansohn from the electric organ of the electric eel.

Study of cholinesterase activity in various tissues has revealed that cholinesterase is not a single enzyme but rather a name for a group of enzymes of varying characteristics and biological significance. One classification groups the cholinesterases into two broad classes, the "true" cholinesterases and the "pseudo" cholinesterases. The "true" enzymes are represented by the cholinesterases of nervous tissue, red blood cells, and the electric organ of the electric eel and similar marine species. The cholinesterases of the blood plasma of most (but not all) animal species and of tissues other than nervous tissue belong to the class of "pseudo" cholinesterases.

The two classes of cholinesterases are distinguishable from each other in terms of substrate specificity and biological occurrence and function. Thus, though both red cell ("true") and plasma ("pseudo") cholinesterase will act upon acetylcholine, other choline esters (propionylcholine, benzoylcholine) are much less readily hydrolyzed by the red-cell enzyme than is acetylcholine; whereas the reverse is true for the plasma enzyme, which will, for example, rapidly decompose benzoylcholine. Distinction between the two types of cholinesterases is frequently based upon such differences in substrate specificity. Furthermore, the function of the cholinesterase of nervous tissue appears to be understandable in relation to the production and disappearance of acetylcholine in nerve function, whereas the plasma enzyme is one of the plasma proteins whose precise physiological function is not yet clear.

Interest in cholinesterases has been heightened by the discovery that drugs of the physostigmine (eserine) class, together with certain commercially important insecticides, owe their mechanism of action to the ability to inactivate cholinesterase. Such anticholinesterase action can be demonstrated *in vivo* and *in vitro*. In the case of physostigmine, inactivation of the enzyme appears to be based upon competitive inhibition, the drug competing with acetylcholine for, and blocking it from, access to the enzyme molecule. This action is reversible in the laboratory by dialysis, enzymatic activity being restored when the drug is dialyzed away. In the case of patients, the cholinesterase activity of blood and tissues is lowered considerably after treatment with the drug, but returns to normal in a matter of hours.

An anticholinesterase drug which irreversibly[1a] inactivates cholinesterase is illustrated by the substance di-isopropylfluophosphate (DFP).

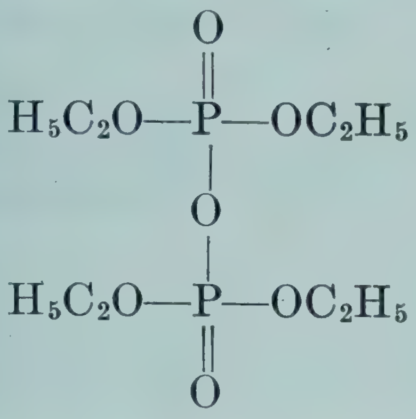


Di-isopropylfluophosphate
(DFP)

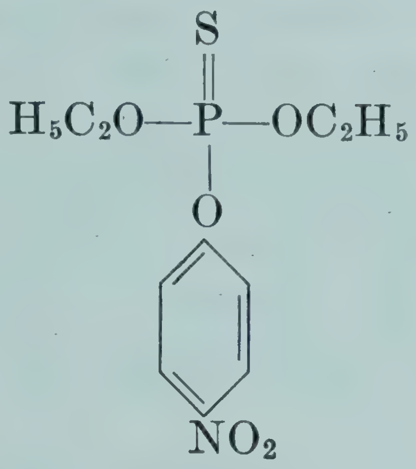
DFP is about 100 times as effective as physostigmine in anticholinesterase potency *in vitro;* furthermore, the reaction of drug and enzyme is irreversible. It has been shown that DFP reacts directly with the enzyme through the P—F linkage, the F splitting off in the process and the remainder of the DFP molecule becoming attached to the enzyme, giving an enzymatically inactive product. Exactly how and why the DFP reacts with the polypeptide chain of the enzyme protein is not clear. Clinically, DFP is used in the treatment of glaucoma, paralytic ileus, and other conditions involving muscular dysfunction. Decrease in cholinesterase activity produced by DFP treatment in patients is much more prolonged than that produced by physostigmine, and essentially dependent upon the replacement of inactivated enzyme by new enzyme.

[1a] Irreversible is used here in the sense that the inactivation cannot be reversed by dialysis, the drug being firmly bound to the enzyme. However, Wilson (*J. Biol. Chem.*, **190**, 111 (1951); *J. Am. Chem. Soc.*, 75, 4628 (1953)) has shown that treatment of the inactive enzyme with solutions of hydroxylamine and similar compounds will lead to the gradual restoration of enzymatic activity in vitro. The bound inactivating drug is presumably split from the enzyme molecule by this treatment.

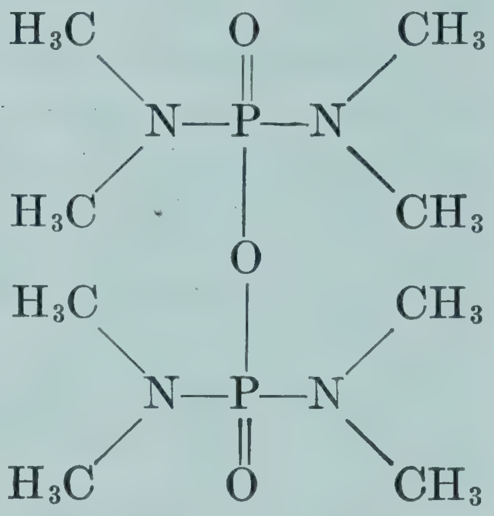
Insecticides whose mechanism of action is ascribed to anticholinesterase activity include the following:



Tetraethyl pyrophosphate (TEPP)



Parathion



Octamethyl pyrophosphoramide (OMPA)

In the case of TEPP, insecticidal activity is due to a direct action on cholinesterase, TEPP being similar to DFP in this respect. Parathion does not have any significant anticholinesterase activity when quite pure; insecticidal activity may be due to impurities, or to metabolic conversion of the P═S bond to P═O, which produces a substance (Para-oxon) with strong anticholinesterase activity. OMPA is another example of a compound which has no anticholinesterase activity as such, but is converted by plant and animal tissues into a potent anticholinesterase, probably by oxidation within the cell, although the chemical nature of the reaction is not yet entirely clear.

EXPERIMENTS ON THE LIPIDES OF NERVOUS TISSUE

1. *Preparation of Pure Lecithin.*[2] Free 4 pounds of brain tissue from adhering foreign tissue and mince in a chopping machine. Dry in a vacuum drying oven. (If such an oven is not available dry the tissue by treating several times with cold acetone.) Extract twice with acetone, using about 1.6 liters. Extract the residue with 2.4 liters of hot alcohol. Concentrate to one-third the original volume. Put in the refrigerator at 0° C. overnight. Filter. To the filtrate add a cold saturated solution of cadmium chloride sufficient to completely precipitate the lecithin.

To avoid decomposition in purification do not use high temperatures when effecting solution or concentration, and reduce the use of water to a minimum. The principal impurity in the cadmium precipitate is cephalin.

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[2] The preparation from egg yolk is somewhat simpler. Stir the fresh egg yolks to a homogeneous emulsion with an egg-beater. Strain through cheesecloth. Pour into a double volume of hot 95 per cent alcohol. Allow to cool. Precipitate with cadmium chloride and purify as indicated above.

To remove this, shake the salts well with ether, and centrifuge. Repeat 8 to 10 times. Suspend 10 g. of the salts in 40 ml. of chloroform, and shake at room temperature until a slightly opalescent solution is formed. Add a cold 25 per cent solution of ammonia gas in 95 per cent methyl alcohol as long as a precipitate is formed, avoiding a large excess. Centrifuge. Concentrate at about 10 to 15 mm. pressure on a water bath at 35° to 40° C. Dissolve in dry ether and concentrate as before to get rid of moisture. Repeat. Dissolve in a minimum amount of ether and pour into acetone (about 50 ml.). Decant. Dry the residue in a vacuum desiccator over H_2SO_4 and keep in a sealed glass tube to prevent oxidation.

2. *Preparation of Crude Lecithin.*[3] Treat the finely divided brain of a sheep or a rabbit with enough ether to cover well, and allow it to stand in the cold for 48 to 72 hours. The cold ether will extract lecithin and cholesterol. Filter and add two volumes of acetone to the filtrate to precipitate the lecithin. Filter off the lecithin, saving the filtrate for the preparation of cholesterol (see below) if desired. Test the lecithin as follows:

(a) MICROSCOPICAL EXAMINATION. Suspend a small portion in a drop of water on a slide and examine under the microscope. Do you see any "myelin forms"?

(b) OSMIC ACID TEST.[4] Treat a small portion with osmic acid. What happens? Why?

(c) ACROLEIN TEST. Make the acrolein test according to the directions on p. 106.

(d) TEST FOR PHOSPHORUS. See p. 212.

3. *Preparation of Cephalin.* Free 4 pounds of brain tissue from membranes, mince in a hashing machine, and dry in a vacuum drier. Pulverize and dry further. Extract exhaustively with acetone, using about 2 liters. Free from acetone in the vacuum drier and extract with about 3 liters of 95 per cent alcohol. Extract the residue with 2 liters of ether. Concentrate to a small volume. Let stand at 0° C. over night. Centrifuge and pour supernatant liquid into 98.5 per cent alcohol warmed to 60° C. Dissolve the precipitate in ether. Allow it to stand at 0° C. over night. Repeat the precipitation with alcohol, redissolving in ether until on standing the ethereal extract no longer deposits a sediment of white matter. The final precipitate is dried and preserved as for lecithin.

4. *Preparation of Cholesterol.*[5] Place a small amount of finely divided brain tissue under ether and stir occasionally for one hour. Filter, evaporate the filtrate to dryness on a water bath, and test the cholesterol according to the directions given below. (If it is desired, the ether-acetone filtrate from the lecithin may be used for the isolation of cholesterol. In these cases it is simply necessary to evaporate the solution to dryness on a water bath.) Upon the cholesterol prepared by either of the above methods make the following tests:

(a) MICROSCOPICAL EXAMINATION. Examine the crystals under the microscope and compare them with those in Fig. 101.

(b) H_2SO_4 TEST (SALKOWSKI). Dissolve a few crystals of cholesterol in a little chloroform and add an equal volume of concentrated sulfuric acid. A play of colors from bluish-red to cherry-red and purple is noted in the chloroform, while the acid assumes a marked green fluorescence.

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[3] The lecithin prepared in this way is satisfactory for the qualitative tests.

[4] Osmic acid serves to detect fats which contain unsaturated fatty acid radicals—e.g., oleic acid—in their molecule.

[5] Pure cholesterol may be prepared from gallstones. Extract with benzene. Evaporate Recrystallize from alcohol.

(c) ACETIC ANHYDRIDE-H_2SO_4 TEST (LIEBERMANN-BURCHARD). Dissolve a few crystals of cholesterol in 2 ml. of chloroform in a dry test tube. Now add 10 drops of acetic anhydride and 1 to 3 drops of concentrated sulfuric acid. The solution becomes red, then blue, and finally bluish-green in color.

(d) FORMALDEHYDE-H_2SO_4 TEST. To 2 ml. of a chloroform solution in a dry test tube add 2 ml. of formaldehyde-H_2SO_4 solution (1 part of 40 per cent formaldehyde to 50 of the acid). Note the cherry color in the chloroform. Pour off the chloroform into another tube and add 2 to 3 drops of acetic anhydride. Note the blue color. This test is said to be more delicate than Salkowski's test.

5. *Preparation of Glycolipide.* Mince 100 g. of sheep brains. Transfer to a 500-ml. flask and add slowly with shaking 200 ml. of alcohol. Heat on a water bath for one hour with occasional shaking. Filter hot. Treat the residue on the water bath with another 100 ml. of alcohol for 15 minutes. Filter, and combine the filtrates. Let stand over night. Filter off the precipitate on a small paper. Transfer the paper to a beaker containing 50 ml. of hot alcohol. Stir to dissolve the lipide and filter hot. Let cool. Filter or centrifuge, and wash the precipitate with ether to remove cholesterol. The residue consists mainly of the glycolipides phrenosin and kerasin.

(a) MICROSCOPICAL EXAMINATION. Suspend a small portion in a drop of water on a slide and examine under the microscope.

(b) SOLUBILITY. Try the solubility of the glycolipide in water, and in dilute acid and alkali, and in hot and cold alcohol and hot and cold ether.

(c) PHOSPHORUS. Test for phosphorus according to the directions on p. 212. How does the result compare with that obtained with lecithin?

(d) Place a little glycolipide on platinum foil and warm. Note the odor.

(e) HYDROLYSIS OF GLYCOLIPIDE. Place the remaining glycolipide in a small evaporating dish, add equal volumes of water and dilute hydrochloric acid, and boil for one hour. Cool, neutralize with solid sodium carbonate, filter, and test with Benedict's solution. Is there any reduction, and if so how do you explain it?

6. *Tests for Choline.*

(a) ROSENHEIM'S PERIODIDE TEST. Prepare an alcoholic extract of the fluid under examination, and after evaporation apply Rosenheim's iodo-potassium iodide solution[6] to a little of the residue. In a short time, dark brown plates and prisms of choline periodide begin to form and may be detected by means of the microscope. Occasionally they are large enough to be visible to the naked eye. They somewhat resemble crystals of hemin (see p. 483). If the slide be permitted to stand, thus allowing the fluid to evaporate, the crystals will disappear and leave brown oily drops. They will reappear, however, upon the addition of fresh iodine solution. v. Staněk claims that this choline compound has the formula $C_5H_{14}NOI \cdot I_8$.

(b) ROSENHEIM'S BISMUTH TEST. Extract the fluid under examination with absolute alcohol, evaporate, and re-extract the residue. Repeat the extraction several times. Dissolve the final residue in 2 to 3 ml. of water and add a drop of Kraut's reagent.[7] Choline is indicated by the appearance of a bright brick-red precipitate.

[6-7] See Appendix.

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12

Enzymes and Their Action: Cell Respiration

The myriad chemical transformations going on continually in living matter would not be possible without enzymes, which are the most important tools of the living cell. For the hydrolysis or the oxidation of such substances as fats and proteins, in the laboratory we commonly employ strong acids or alkalies or oxidizing agents and high temperatures. Such reagents and temperatures are incompatible with the existence of living matter. The cells are, however, able to carry out such reactions at approximate neutrality, at body temperatures, and at high speed with the aid of enzymes. As an example it has been shown that it takes 10,000,000 times as great a concentration of hydrogen ion as of the enzyme sucrase to decompose a given amount of cane sugar in a given time at body temperature.

Enzymes are organic catalysts produced by living organisms. They are generally soluble and colloidal substances, characterized by great activity, specificity, and susceptibility to the influence of pH, of temperature, and of other environmental changes.

Catalysis. As indicated, enzymes act catalytically. Catalysts are substances that alter the speed of chemical reactions without themselves undergoing any permanent change. This definition indicates that catalysts do not initiate chemical reactions but only speed up reactions already proceeding at a slow rate. Thus hydrogen peroxide undergoes a very slow spontaneous decomposition at room temperature with the formation of water and oxygen. The addition of a little finely divided platinum or of an enzyme called catalase enormously increases the rate of this decomposition. Catalysts function in many reactions, however, which cannot definitely be shown to be proceeding in their absence. It may be assumed in these cases that the reaction is actually proceeding but at an immeasurably slow rate. From the practical standpoint, however, the catalyst or enzyme in these cases does initiate a reaction.

Inorganic catalysts are of great importance in industry. Thus sulfur dioxide reacts slowly with oxygen to form sulfur trioxide. If, however, oxides of nitrogen are present, they catalyze the reaction so that it proceeds much more rapidly, e.g.,

$$NO + \tfrac{1}{2}O_2 = NO_2$$
$$SO_2 + NO_2 = SO_3 + NO$$

Further analogies between enzymes and inorganic catalysts will be mentioned later.

Classification. All the enzymes which have been isolated in pure condition to date are proteins. The properties which are used to classify proteins are much too general to be of use in classifying enzymes. Hence it is customary to classify them according to their action. Some enzymes contain characteristic prosthetic groups conjugated with the protein, and these groups are also used for further classification. For example, oxidizing enzymes are commonly subdivided according to whether they contain heme, riboflavin, etc., as prosthetic groups. A considerable number of enzymes can be classified under the general term *transferases* since their action is to transfer such groups as amino, methyl, acetyl, glucosyl, fructosyl, or phosphoryl from one compound to another.

HYDROLASES. A great many enzymes catalyze hydrolysis reactions. This large group is commonly designated as hydrolases. The hydrolases, like other enzymes, are further classified according to their substrates, i.e., the substances on which they act. The nomenclature is harmonized as far as possible by using the termination *ase* in the names of enzymes. Thus we have esterases hydrolyzing esters, carbohydrases hydrolyzing carbohydrates, proteinases hydrolyzing proteins, amidases hydrolyzing amides, etc. Under each of these groups are individual enzymes which act on specific substrates, such as maltase which splits maltose, lactase which hydrolyzes lactose, etc. The source of the enzyme may also be indicated in its name, such as salivary amylase, pancreatic lipase, etc. Some of the old names for enzymes are still used as a matter of convenience—such names, for example, as pepsin and trypsin.

OXIDIZING ENZYMES. Another large group of enzymes catalyzes oxidations. These may be called oxidizing enzymes. Oxidizing enzymes are frequently divided into two groups—oxidases and dehydrogenases—which are distinguished by their mode of action. This is not an entirely satisfactory division since some enzymes can be placed in either group. A more suitable classification makes no attempt to distinguish between oxidases and dehydrogenases—other criteria are used instead. For example, the following groups may be recognized: (1) Enzymes containing iron, (2) enzymes containing copper, (3) enzymes containing coenzymes I or II, (4) enzymes which catalyze the reduction of cytochrome, (5) enzymes which contain riboflavin (yellow enzymes), and (6) various other oxidizing enzymes.

There are still other enzymes that do not fit into either of these groups. For example, phosphorylases act similarly to hydrolases, except that a phosphoric acid residue, and not water, is used in splitting the substrate. There are other enzymes, the hydrases, which remove or add water to the substrate without hydrolyzing it, and there are still other enzymes, the true desmolases, which catalyze the splitting of a carbon chain. Thus, zymohexase catalyzes the reversible splitting of fructose-1,6-diphosphate into the triosephosphates, dihydroxyacetone phosphate and phosphoglyceric aldehyde. The mutases catalyze the oxidation of one molecule of the substrate and the simultaneous reduction of another molecule of the substrate. Recently aldehyde mutase has been shown by Racker to be nothing more than a mixture of alcohol dehydrogenase and aldehyde dehydrogenase.

In a number of cases, two or more names are commonly applied to the same enzyme. For example, amylase is also known as diastase; sucrase is commonly called invertase or saccharase; cytochrome oxidase was formerly called indophenol oxidase, etc. Sometimes such synonyms arose because enzymes from different sources at first were not recognized to be identical. For example, tyrosinase or monophenoloxidase was at one time thought to be distinct from polyphenoloxidase. In other cases the original name given to an enzyme has proved to be inadequate. For example, the name zymase was at one time used for the yeast enzyme which ferments sugars to alcohol. Subsequently it was found that the original zymase was really several enzymes and the original name had to be discarded.

A list of some of the more important enzymes is given in this connection, without any attempt to adhere to a rigid classification (see Table, pp. 306–307).

Chemical Nature. The problem of the purification of enzymes has been essentially one of their separation from other associated proteins. Because of the difficulties of such separation and purification, the chemical nature of enzymes has been much in doubt. The first preparation of a crystalline enzyme was that of urease by Sumner in 1926 (see Fig. 75). Since that time crystalline pepsin was obtained by Northrop (see Fig. 76), crystalline trypsin and chymotrypsin by Northrop and Kunitz, and pepsinogen by Herriott and Northrop. Crystals of yellow respiratory ferment were obtained by Warburg, and those of β-amylase (Fig. 74) by Balls. To date, about 60 enzymes have been obtained in crystalline form. However, a few of the claims made are either erroneous or dubious. All of these crystalline preparations have proved to be proteins, and the specific action of these enzymes has never been observed in the absence of the specific proteins. There is additional evidence which shows rather clearly that the enzymes and crystalline proteins are identical. If the protein is destroyed or denatured, the enzyme action is lost. Also, various crystalline enzyme preparations have been tested for homogeneity, using such criteria as diffusion, movement in an ultracentrifuge, migration in an electrical field, solubility, etc.; and with the exception of pepsin each has been found to consist of a single component. Pepsin appears to be a mixture of similar proteins. Some of these crystalline enzymes have been recrystallized as many as ten times without change in composition and properties.

Further evidence that the enzyme itself is a protein has been presented, as in the case of pepsin, by showing that if the protein of the material is denatured through the action of alkali, the activity of the enzyme is decreased in a parallel manner. For example, pepsin becomes completely denatured and inactive at pH 10.5. If the solution is adjusted to pH 5.4 and is allowed to stand at this pH, a small part of the protein is renatured and there is a corresponding appearance of activity of the enzyme. The solution should be adjusted to about pH 1 before testing for the enzyme activity.

Crystalline trypsin denatured by heat also shows on standing a parallel reappearance of activity and of native protein in the solution. The trypsin

CLASSIFICATION OF ENZYMES

Name and Class	*Distribution*	*Substrate*	*End-products*
Hydrolases			
Carbohydrases		Carbohydrates	
1. Amylase	Pancreas, saliva, malt, etc.	Starch, dextrin, etc.	Maltose and dextrins
2. Lactase	Intestinal juice and mucosa	Lactose	Glucose and galactose
3. Maltase	Intestinal juice, yeast, etc.	Maltose	Glucose
4. Sucrase	Intestinal juice, yeast, etc.	Sucrose	Glucose and fructose
5. Emulsin	Plants	β-Glucosides	Glucose, etc.
Nucleases		Nucleic acid and derivatives	
1. Polynucleotidase	Pancreatic juice, intestinal juice, etc.	Nucleic acid	Nucleotides
2. Nucleotidase	Intestinal juice, and other tissues	Nucleotides	Nucleosides and phosphoric acid
3. Nucleosidase	Animal tissues	Nucleosides	Carbohydrate and bases
Amidases		Amino compounds and amides	
1. Arginase	Liver	Arginine	Ornithine and urea
2. Urease	Bacteria, soybean, jack bean, etc.	Urea	Carbon dioxide and ammonia
3. Glutaminase	Liver, etc.	Glutamine	Glutamic acid and ammonia
4. Transaminase	Animal tissues	Glutamic acid and oxalacetic acid, etc.	α-Ketoglutaric acid, aspartic acid, etc.
Purine Deaminases		Purine bases and derivatives	
1. Adenase	Animal tissues	Adenine	Hypoxanthine and ammonia
2. Guanase	Animal tissues	Guanine	Xanthine and ammonia
Peptidases		Peptides	
1. Aminopolypeptidase	Yeast, intestines, etc.	Polypeptides	Simpler peptides and amino acids
2. Carboxypeptidase	Pancreas	Polypeptides	Simpler peptides and amino acids
3. Dipeptidase	Plant and animal tissues and bacteria	Dipeptides	Amino acids
4. Prolinase	Animal tissues and yeast	Proline peptides	Proline and simpler peptides
Proteinases		Proteins	
1. Pepsin	Gastric juice	Proteins	Proteoses, peptones, etc.
2. Trypsin	Pancreatic juice	Proteins, proteoses, and peptones	Polypeptides and amino acids
3. Cathepsin	Animal tissues	Proteins	Proteoses and peptones
4. Rennin	Calf stomach	Casein	Paracasein
5. Chymotrypsin	Pancreatic juice	Proteins, proteoses, and peptones	Polypeptides and amino acids
6. Papain	Papaya, other plants	Proteins, proteoses, and peptones	Polypeptides and amino acids
7. Ficin	Fig sap	Proteins	Proteoses, etc.
Esterases		Esters	Alcohols and acids
1. Lipase	Pancreas, castor bean, etc.	Fats	Glycerol and fatty acids
2. Esterases	Liver, etc.	Ethyl butyrate, etc.	Alcohols and acids
3. Phosphatases	Plant and animal tissues	Esters of phosphoric acid	Phosphate and alcohol
4. Sulfatases	Animal and plant tissues	Esters of sulfuric acid	Sulfuric acid and alcohol
5. Cholinesterase	Blood, tissues	Acetylcholine	Choline and acetic acid
Iron Enzymes			
1. Catalase	All living organisms except a few species of microörganisms	Hydrogen peroxide	Water and oxygen
2. Cytochrome oxidase	All living organisms except a few species of microörganisms	Reduced cytochrome C in the presence of oxygen	Oxidized cytochrome C and water
3. Peroxidase	Nearly all plant cells	A large number of phenols, aromatic amines, etc., in the presence of H_2O_2	Oxidation product of substrate and water
Copper Enzymes			
1. Tyrosinase (polyphenoloxidase, monophenoloxidase)	Plant and animal tissues	Various phenolic compounds	Oxidation product of substrate
2. Ascorbic acid oxidase	Plant tissues	Ascorbic acid in the presence of oxygen	Dehydroascorbic acid

CLASSIFICATION OF ENZYMES—(*Continued*)

Name and Class	*Distribution*	*Substrate*	*End-products*
Enzymes Containing Coenzymes I *and/or* II			
1. Alcohol dehydrogenase	Animal and plant tissues	Ethyl alcohol and other alcohols	Acetaldehyde and other aldehydes
2. Malic dehydrogenase	Animal and plant tissues	L(−) Malic acid	Oxalacetic acid
3. Isocitric dehydrogenase	Animal and plant tissues	L-Isocitric acid	Oxalosuccinic acid
4. Lactic dehydrogenase	Animal tissues and yeast	Lactic acid	Pyruvic acid
5. β-Hydroxybutyric dehydrogenase	Liver, kidneys, and heart	L-β-Hydroxybutyric acid	Acetoacetic acid
6. Glucose dehydrogenase	Animal tissues	D-Glucose	D-Gluconic acid
7. Robison ester dehydrogenase	Erythrocytes and yeast	Robison ester (hexose-6-phosphate)	Phosphohexonic acid
8. Glycerophosphate dehydrogenase	Animal tissues	Glycerophosphate	Phosphoglyceric acid
9. Aldehyde dehydrogenase	Liver	Aldehydes	Acids
Enzymes Which Reduce Cytochrome			
1. Succinic dehydrogenase (as ordinarily prepared)	Plants, animals, and microörganisms	Succinic acid	Fumaric acid
Yellow Enzymes			
1. Warburg's old yellow enzyme	Yeast	Reduced coenzyme II	Oxidized coenzyme II and reduced yellow enzyme
2. Diaphorase	Bacteria, yeasts, higher plants, and animals	Reduced coenzyme I	Oxidized coenzyme I and reduced diaphorase
3. Haas enzyme	Yeast	Reduced coenzyme II	Oxidized coenzyme II and reduced yellow enzyme
4. Xanthine oxidase	Animal tissues	Hypoxanthine, xanthine, aldehydes, reduced coenzyme I, etc.	Xanthine, uric acid, acids, oxidized coenzyme I, etc. In presence of air, H_2O_2.
5. D-Amino acid oxidase	Animal tissues	D-Amino acids + O_2	α-Keto-acids + NH_3 + H_2O_2
6. L-Amino acid oxidases	Animals, snake venom	L-Amino acids	Keto acids and ammonia
7. TPN-Cytochrome C reductase	Yeast, liver	Reduced coenzyme II and cytochrome C	Oxidized coenzyme II and reduced cytochrome C
8. DPN-Cytochrome C reductase	Liver, yeast	Reduced coenzyme I and cytochrome C	Oxidized coenzyme I and reduced cytochrome C
Hydrases			
1. Fumarase	Living organisms in general	Fumaric acid + H_2O	L-Malic acid
2. Aconitase	Animals and plants	Citric acid	*cis*-Aconitic acid and L-isocitric acid
3. Enolase	Animal tissues and yeast	2-Phosphoglyceric acid	Phosphopyruvic acid + H_2O
Mutases			
1. Glyoxalase	Living organisms in general	Methyl glyoxal and other substituted glyoxals	D(−)Lactic acid
Desmolases			
1. Zymohexase (aldolase)	All cells	Fructose-1,6-diphosphate	Dihydroxyacetone phosphoric acid and phosphoglyceric acid
2. Carboxylase	Plant tissues	Pyruvic acid	Acetaldehyde and CO_2
3. β-Keto carboxylases	Animals, bacteria, plants	β-Keto acids	α-Keto acids
4. Amino acid decarboxylases	Plants, animals, bacteria	L-Amino acids	Amines and CO_2
5. Carbonic anhydrase	Erythrocytes	Carbonic acid	CO_2 + H_2O
Other enzymes			
1. Phosphorylase	Animal and plant tissues	Starch or glycogen and phosphate	Glucose-1-phosphate
2. Phosphohexoisomerase	Animal and plant tissues	Glucose-6-phosphate	Fructose-6-phosphate
3. Hexokinase	Yeast, animal tissues	Adenosinetriphosphate + glucose	Adenosinediphosphate + glucose-6-phosphate
4. Phosphoglucomutase	Plants and animals	Glucose-1-phosphate	Glucose-6-phosphate

may also be hydrolyzed by pepsin in acid solution, and the loss in activity is just proportional to the hydrolysis of the trypsin protein.

In the case of those crystalline enzymes which have been most studied it seems clear that the enzyme activity is bound up with the integrity of the protein of the preparation and that these enzymes are thus properly characterized as proteins. Experiments by Northrop on the acetylation of pepsin suggest that the activity is bound up, not with the primary amino groups, but with the presence of free hydroxyl groups of tyrosine. That certain enzymes may owe their activity to groups of a nonprotein character combined with protein is clear from our knowledge of the oxidases, some of which appear to be heme-protein derivatives, the activity

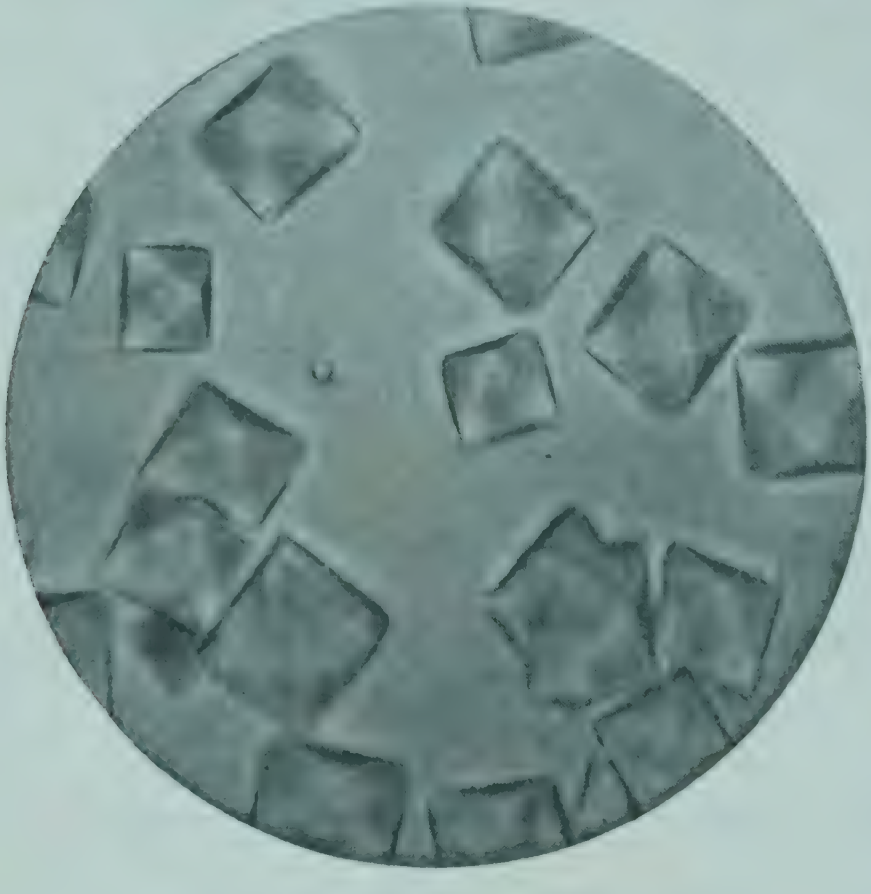


FIG. 74. β-AMYLASE CRYSTALS.
Courtesy, Dr. A. K. Balls.

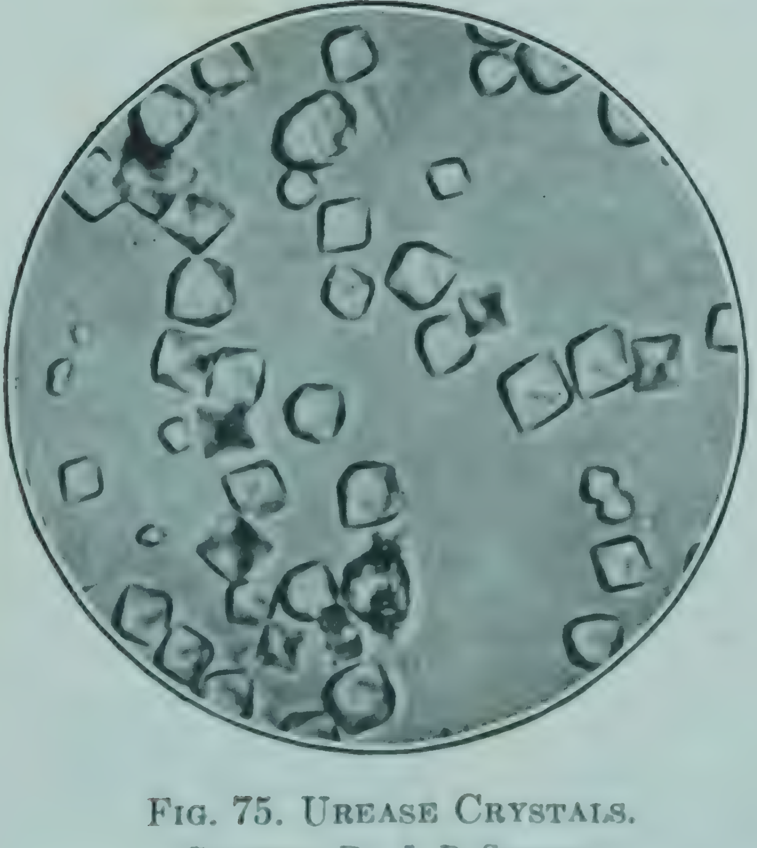


FIG. 75. UREASE CRYSTALS.
Courtesy, Dr. J. B. Sumner.

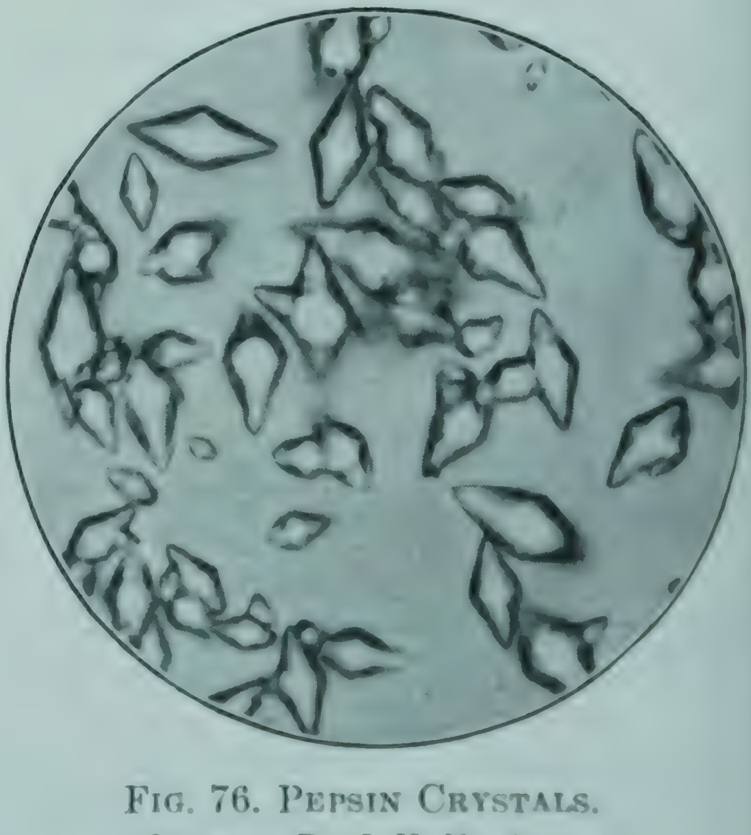


FIG. 76. PEPSIN CRYSTALS.
Courtesy, Dr. J. H. Northrop.

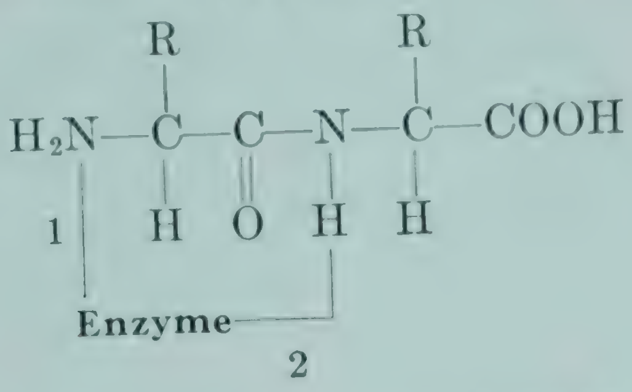
of which is bound up with the iron-pyrrole group. Peroxidase is a specific example of such an oxidase. Heme has, however, much less peroxidase activity than its natural protein combinations, so that both elements are evidently essential for proper function. Preparations of certain other enzymes—such as amylase, sucrase, and lipase—have been variously reported as protein-free or very low in protein. Protein tests made on enzyme solutions are, however, inconclusive, since the protein tests are far less sensitive than the tests for enzyme activity. As to the nature of the groups in the enzyme molecules which are responsible for the specificity of their action, almost nothing is known except in the cases of some of the oxidases.

Specificity and Mechanism of Enzyme Actions. Not only do proteinases not act upon carbohydrates, nor carbohydrases upon proteins, but much greater degrees of specificity exist. Thus maltose, lactose, and sucrose require different enzymes for their hydrolysis. Further, the maltase of yeast splits maltose (an α-glucoside) and certain other α-glucosides, but not β-glucosides; but β-glucosidase or emulsin hydrolyzes only β-glucosides. The maltase will not hydrolyze any glucosides in which the α-glucose residue has been altered. The specificity appears to depend primarily on the glucose residue and secondarily on the group attached to it. The dependence of enzyme action on the configuration of the substrate is further brought out by the fact that dipeptidase will attack glycyl-L-leucine but not glycyl-D-leucine. It was findings of this sort that led Emil Fischer to suggest that the relation of enzyme to substrate was much like that of a key to a lock.

It has been pointed out that enzymes are catalysts. Our knowledge of the mechanisms involved in enzymic catalyses is, however, very slight. There is much evidence that enzymes form intermediate compounds with the substrates on which they act. This intermediate compound is believed to be much less stable than the original substrate so that the latter now breaks down spontaneously, the enzyme being again liberated. According to the views of some workers there may be two groups (or perhaps more) in the enzyme which are involved, one group acting chiefly to bind the enzyme with the substrate while the second combines with another part of the substrate so as to reduce the stability of the substrate. This view has been developed especially in connection with the peptidases.

Intestinal dipeptidase will split all peptides made up of naturally occurring amino acids. It will not split peptides containing the optical isomers of these amino acids. Thus glycyl-L-leucine will be split but not glycyl-D-leucine. If the free carboxyl group of the dipeptide is esterified (as by making the ethyl ester), hydrolysis still occurs, indicating that the enzyme does not combine with the carboxyl group. If the free amino group is acetylated, the compound is not split. This indicates that the enzyme combines with the amino group of the peptide. If the H of the NH group of the peptide is substituted, it is not acted upon. Replacement of the H of the adjacent C atoms also prevents splitting.

These observations indicate that the enzyme must have at least four groups showing a similar space relationship to these four groups in the dipeptide. It has been suggested further that the enzyme combines with the dipeptide first through the amino group and then a second group in the enzyme attaches to the NH group, this latter linkage leading to a lowered stability of the peptide linkage so that splitting occurs. It has been shown that acetylation of the NH group in peptides does lead to instability. The findings may be summarized diagrammatically as follows. It must be understood, however, that no explanation of the mechanism of such enzyme actions is yet established.



$$H_2N-\underset{H}{\overset{R}{C}}-\underset{O}{\overset{\|}{C}}-\underset{H}{N}-\underset{H}{\overset{R}{C}}-COOH$$

Keilin and Hartree observed that peroxidase forms compounds with hydrogen peroxide that show definite absorption bands. Chance has investigated these compounds, also compounds of catalase with hydrogen peroxide. The reaction of catalase with hydrogen peroxide is:

(1) $Cat(OH)_4 + H_2O_2 = Cat(OH)_3OOH + H_2O$

(2) $Cat(OH)_3OOH + H_2O_2 = Cat(OH)_4 + H_2O + O_2$

The reactions with peroxidase would be:

(1) $PerOH + H_2O_2 = PerOOH + H_2O$

(2) $PerOOH + AH_2$ (oxidizable substance) $= PerOH + H_2O + A$

Action of Physical and Chemical Agents on Enzymes. Variations of temperature and of hydrogen-ion concentration of the medium have a marked effect on enzyme action. The speed of action must naturally also vary with the concentration of the enzyme and the concentration of the substrate. The effects of activators, of coenzymes, and of a proper concentration of electrolyte are of importance in many cases, as well as the negative effects of inhibitors or "poisons." As digestion proceeds, the products of the reaction may have an inhibiting effect.

Influence of Temperature. Most enzymes are inactivated by heating their solutions to temperatures in the neighborhood of 60° C. This inactivation is associated with a denaturation of the protein of the preparation. In some cases there is, on standing, an appreciable reversal of the denaturation process with the reappearance of some enzyme activity. Heat resistance may be influenced by the presence of protective substances, such as the enzyme substrate, or by pH. Trypsin is more resistant in acid than in alkaline solutions and is in fact very resistant. In acid solution it may endure temperatures near the boiling point. Under these conditions it is denatured, but the process is rapidly reversed on cooling. Temperatures as low as freezing do not commonly destroy enzymes.

Within their active range most enzymes have a temperature coefficient (Q_{10}) of about 2; i.e., they approximately double their activity for each 10 degrees C. rise in temperature. As the temperature rises, however, inactivation influences the result, since inactivation also increases with rise in temperature. The optimum temperature for the action of an enzyme is thus determined by the balance between increase of activity with temperature and decrease in amount of enzyme present due to inactivation. In prolonged experiments, enzyme destruction becomes more important than in short experiments, and the optimum temperature for such experiments is therefore lower. The majority of enzymes act most rapidly at 40° to 50° C. For certain plant proteinases the optimum is higher.

Influence of Hydrogen-ion Concentration. Enzymes are inactivated by certain degrees of acidity or alkalinity. Thus yeast sucrase is inactivated rapidly below pH 3 and pepsin above pH 8. By the optimum pH of an enzyme is meant the pH at which it shows greatest activity. For most enzymes this point lies between pH 4 and 8, and for many between pH 5 and 7. For practically all enzymes the pH range is rather narrow and activity falls off rapidly on both sides of the optimum. The best pH depends to some extent on temperature and enzyme and substrate concentrations. The nature of the buffer may change the optimum pH of an enzyme considerably. The same is true for the nature of the substrate. When the substrates are ionizable, as in the case of proteins, the optimum pH may vary with the substrate. This is true for pepsin. One of the lowest pH optima is that for pepsin (pH 2); and one of the highest, that for blood phosphatase (pH 9). Alterations in pH may affect the dispersion of the enzyme, the rapidity of combination of enzyme and substrate, and the decomposition of the enzyme-substrate compelx with the formation of reaction products.

Influence of Other Physical Agents. Enzymes may be destroyed by shaking, by ultraviolet irradiation, and by x-rays. These agents probably act by denaturing the enzyme.

Influence of Chemical Agents. Most enzymes when acting at optimum temperatures will show, on the addition of chemical agents, either an inhibiting effect or no effect at all. In some cases, however, an acceleration is noted, and certain enzyme preparations are inactive unless additions are made.

If salivary amylase is dialyzed free from NaCl, it becomes inactive on starch. but activity is restored on the addition of sodium, potassium, or certain other chlorides. The chloride here may be called a coenzyme; i.e., it belongs to a class of substances which are specific for particular enzymes and necessary for the activity of those enzymes. In this sense, phosphate, which is essential for the action of zymase, is also a coenzyme, as is the organic crystalloidal substance cozymase which is also necessary for zymase action. More recent usage limits the term coenzyme to organic compounds which act in this manner, e.g., diphosphopyridine nucleotide.

Many enzymes are activated by such metallic ions as Mg^{++}, Co^{++}, Mn^{++}, Zn^{++}, etc. Emil Smith[1] has studied the activation of various peptidases and believes that at least two of the bonds between substrate and enzyme are through the metallic ion.

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[1] Sumner and Myrbäck: *The Enzymes*, New York, Academic Press Inc., 1950, Vol. 1. Part 2, p. 793.

Activators and kinases are believed to bring about chemical changes in the inactive forms of enzymes, so as to render them active. Thus HCl changes inactive pepsinogen to active pepsin. The pepsinogen here is called a *zymogen* or *proenzyme*, and the HCl (or more strictly the H ion) is spoken of as an activator. Activators of an organic character are sometimes called kinases. Thus trypsinogen is changed to active trypsin by enterokinase. Enterokinase is probably an enzyme. It can be replaced by trypsin; the action of trypsin upon trypsinogen, therefore, is autocatalytic. Chymotrypsinogen and procarboxypeptidase also are activated by trypsin.

Enzymes are poisoned by a variety of substances which form compounds with them. A number of heavy metals stop enzyme action, apparently by combining with the enzyme acting as an acid, though combination with sulfhydryl groups in enzymes may also be involved. Urease is sensitive to traces of heavy metals. H_2S may combat this toxicity of the heavy metals, probably by combining with them.

HCN and H_2S have little effect on most hydrolytic enzymes. The proteinase called papain is, however, activated by them, and the action of keratinase is much facilitated by the presence of H_2S. Certain oxidases are inactivated by these substances in small amounts, apparently because they combine with the active iron of these enzymes.

Certain acids, such as phosphotungstic or picric acid, combine with the enzyme, acting as a base, to form inactive compounds.

Antienzymes include various naturally occurring inhibitors with some degree of specificity. The blood serum contains antitrypsin, and the mucosa of the intestinal tract appears to contain some antipepsin and antitrypsin. In addition there are various antienzymes which are true immune bodies produced by the usual methods for producing antibodies. For example, antiluciferase, antiurease, and anticatalase have been produced by injecting the appropriate enzymes into animals.

Kinetics of Enzyme Action. Enzyme kinetics deals with the velocity of enzyme reactions and with agents or factors which affect this velocity.

It is usually possible to cause the velocity of an enzyme-catalyzed reaction to remain constant for a sufficient period of time to allow measurement of the enzyme by determining the reaction rate, if enough substrate is used and if other factors such as pH, temperature, etc. are held reasonably constant. A reaction of this variety is called one of apparent zero order and follows an equation of the form

$$\frac{x}{t} = k \qquad \text{or} \tag{1}$$

$$x = kt \tag{1a}$$

where x is the amount of substrate decomposed in time t, $\frac{x}{t}$ is the reaction velocity, and k is a constant depending upon the amount of enzyme present. Provided that factors such as pH and temperature are held constant, k can be used as a measure of the amount of enzyme present.

If the amount of substrate employed is low, as is necessary with enzymes such as catalase which are gradually destroyed by excess substrate, the reaction velocity is often proportional to the amount of substrate present at any time. Such a reaction is called an apparent monomolecular or first-order reaction and follows the equation

$$v = \frac{dx}{dt} = k(A - x) \tag{2}$$

(deduced from simple application of the mass law) where the velocity v must be expressed using the calculus notation $\frac{dx}{dt}$ instead of $\frac{x}{t}$, since v varies continuously with x. The term A signifies the initial concentration of substrate; x is the decrease in concentration of substrate (equivalent to the increase in concentration of products) that has occurred at time t; and k is a constant which varies with the amount of enzyme (at constant pH, temperature, etc.) and can be used as a measure of the amount of enzyme present.

Since Equation (2) is not applicable as it stands, it must be transformed by use of the integral calculus to eliminate the $\frac{dx}{dt}$ term. When this is done the following equation is obtained:

$$\log \frac{A}{(A - x)} = kt, \qquad \text{or} \tag{3}$$

$$k = \frac{1}{t} \log \frac{A}{(A - x)} \tag{3a}$$

If it is desired to obtain the theoretical initial reaction velocity, this can be done by multiplying k by A, or

$$v_i = kA$$

as is apparent from an inspection of Equation (2).

The use of the term *apparent* in describing the order of enzyme-catalyzed reactions is necessary because such reactions usually consist of a sequence of bimolecular reactions, one of which may be rate-limiting and therefore control the observed kinetics. In case water is involved in a rate-limiting bimolecular step, a truly bimolecular reaction will follow monomolecular kinetics, as is explained in texts on physical chemistry, owing to the essentially constant water concentration. Bimolecular kinetics are observed with only a few enzymic reactions and will not be considered here.

In case the concentration of substrate is neither very high nor very low, the observed kinetics of an enzyme-catalyzed reaction may not follow either an apparent zero-order or an apparent first-order reaction. In such a case it is still possible to obtain a valid measure of the amount of enzyme present from kinetic studies if one can measure the length of time necessary to attain decomposition of a certain fixed percentage of the substrate (say 5 per cent). In this case the amount of enzyme is proportional to the reciprocal of the time value.

As has been inferred above, the rate of an enzyme-catalyzed reaction can be affected by factors such as change in temperature, change in pH, and change in substrate concentration (or partial pressure, if a gas is reacting). In addition the enzyme may be inactivated by the substrate or inhibited by various types of enzyme inhibitors.

Temperature change generally results in a bell-shaped curve when enzyme activity is plotted against temperature, with a pronounced optimum. The latter results from an enhancement of reaction rate as the temperature increases, followed eventually by a decrease in rate owing to the denaturation or destruction of the enzyme at higher temperature values. The exact position of the temperature optimum is not of theoretical significance however, since it is dependent upon various factors such as substrate concentration, pH, and the time interval during which the measurement of activity is carried out. It is very difficult to arrive at a good estimate of the initial reaction velocity in determining temperature optima. Hence a somewhat better procedure is to determine the effect of temperature on the enzyme by treating it at various temperatures for a given length of time and then determining activity under standard conditions of pH, temperature, and substrate concentrations. By this procedure an S-shaped curve is obtained which gives a more reproducible measure of the destructive effect of temperature on the enzyme. The effect of temperature on enzymatic activity is of theoretical significance (in calculating energy of activation) in the range where no damage to the enzyme is occurring, and throughout this range enzyme activity generally increases with temperature increase in a predictable way.

Curves showing the effect of pH on enzyme activity in general give optima which are of theoretical significance. Changes in pH may affect primarily the substrate or the enzyme, or both, and are concerned with producing the necessary ionic states of the enzyme or substrate or both.

A fundamental approach to enzyme kinetics was developed by Michaelis and Menten[2], who proposed to measure the affinity of an enzyme for its substrate by studying the effect of substrate concentration on initial reaction velocity with a constant amount of enzyme present. The theoretical treatment is based upon the assumption of the following sequential reactions involving enzyme, substrate, and reaction products:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \quad \overset{k_3}{} \quad E + P \tag{4}$$

where E stands for enzyme, S for substrate, and P for products. The k's are the rate constants for the reactions as indicated by the arrows.

In the simplest case it is assumed that the rate-limiting reaction is $ES \rightarrow E + P$, the formation of ES being much more rapid. By a simple algebraic process, if this assumption is made, the following equation can be derived, which shows the relation between the maximum reaction velocity V_m, obtained when an excess of substrate is present and the enzyme is saturated with substrate, and the reaction velocity v, obtained with substrate concentrations below the saturation level:

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[2] Michaelis and Menten: *Biochem. Z.*, **49**, 333 (1913).

(5) $$v_i = \frac{V_m[S]}{[S] + K_m}$$ or solving for K_m,

(5a) $$K_m = [S]\,\frac{V_m - v_i}{v_i}$$

where v_i is initial reaction velocity for the enzyme-catalyzed reaction in question, $[S]$ is substrate concentration, and K_m, the Michaelis constant, is the *dissociation constant* for the enzyme-substrate complex: i.e., the equilibrium constant for the reaction $ES \rightleftarrows E + S$. In this simple case therefore, $K_m = \frac{k_2}{k_1}$ where the small k's are defined as above. In case the decomposition of the ES complex to yield enzyme + products is not strictly rate-limiting, K_m must be interpreted[3] as $\frac{k_2 + k_3}{k_1}\cdot$ It will be noted that in the simplest case K_m is an inverse measure of the affinity of enzyme for substrate: i.e. the smaller the K_m value, the higher the affinity.

In theory, K_m is equal to the substrate concentration when $v_i = \frac{1}{2}V_m$. However, in determining K_m values, Equations (5) and (5a) are not very suitable, since V_m is not easily obtained. Equation (5) is a branch of an hyperbola, but in practice if an attempt is made to obtain V_m from an estimate of the horizontal asymptote, large errors usually occur for various reasons. However, it is possible, as shown by Lineweaver and Burk,[4] to transform Equation (5) into a linear equation simply by equating the reciprocal of both sides and simplifying by division. Equation (6) is thus obtained:

(6) $$\frac{1}{v_i} = \frac{1}{V_m} + \frac{K_m}{V_m[S]}$$

Here the intercept is $\frac{1}{V_m}$ and the slope is $\frac{K_m}{V_m}\cdot$ There is one objection to this transformation, however; namely, that points representing very low substrate concentrations, which are likely to be inaccurate, are given too great weight, since they become increasingly spread out in the plot of $\frac{1}{v_i}$ against $\frac{1}{[S]}\cdot$ A second type of transformation of the original Equation (5) is better in this respect. It is obtained by multiplying the last equation, (6), by $[S]$ to give:

(6a) $$\frac{[S]}{v_i} = \frac{[S]}{V_m} + \frac{K_m}{V_m}$$

If $\frac{[S]}{v_i}$ is plotted against $[S]$, the slope is $\frac{1}{V_m}$, and the intercept is $\frac{K_m}{V_m}\cdot$ From the intercept and the slope, K_m is therefore readily obtained.

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[3] Briggs and Haldane: *Biochem. J.*, **19**, 338 (1925); Wilson: Chapter in Lardy (ed.): *Respiratory Enzymes*, Minneapolis, Burgess Publishing Co., 1950, p. 17.

[4] Lineweaver and Burk: *J. Am. Chem. Soc.*, **56**, 658 (1934); *Respiratory Enzymes*, *loc. cit.*

The affinity of enzyme for substrate is of the utmost importance and may eventually turn out to be the determining factor in deciding why an enzyme consisting of a protein plus a prosthetic group is so much more efficient as a catalyst than the prosthetic group alone.

Synthetic Action of Enzymes. Enzymic reactions can be divided into two classes:

(1) Exergonic, or those which liberate energy.

(2) Endergonic, or those which require addition of energy in order to occur.

The reactions which result in the synthesis of glucose-1-phosphate from glucose, glutamine from glutamic acid and ammonia, acetylcholine, urea, peptides, riboflavin phosphate, coenzyme I, etc., are endergonic. The energy for these reactions is supplied by the breakdown of adenosine triphosphate.

Enzymic reactions can be divided also into:

(a) Those which proceed to completion.

(b) Those which proceed to a point of equilibrium at which both substrate and products are present in measurable amounts.

Examples of (a) are the decomposition of hydrogen peroxide by catalase, the formation of rhodanate from cyanide and thiosulfate by rhodanese and the synthesis of gum dextran from sucrose by dextran sucrase. An example of (b) is the conversion of glucose-6-phosphate to glucose-1-phosphate by phosphoglucomutase. At equilibrium the digest will contain 5 per cent of glucose-1-phosphate and 95 per cent of glucose-6-phosphate.

One of the most striking demonstrations of the reversibility of enzyme action can be obtained with phosphorylase. This enzyme, which is found in animal tissues as well as tissues of plants, catalyzes the breakdown of glycogen or starch to glucose-1-phosphate:

$$\text{starch} + H_2PO_4^- \rightleftarrows \text{glucose-1-phosphate}$$

The glucose-1-phosphate (Cori ester) can be isolated readily from the system. If glucose-1-phosphate is added to a potato phosphorylase preparation, starch amylose is rapidly formed and can be detected by the color it gives with iodine solution. For this reaction to occur it is necessary to prime the digest by adding a trace of glycogen or starch. Muscle phosphorylase likewise produces starch from glucose-1-phosphate. Phosphorylases prepared from liver, brain, heart, or yeast form glycogen from glucose-1-phosphate. These phosphorylases contain two enzymes, ordinary phosphorylase, which forms 1-4 linkages, and the Q enzyme, which forms 1-6 linkages. The latter is not a true phosphorylase, but a transglucosidase.

The possibility of reversible or synthetic action of enzymes has thus been demonstrated, and it is of the greatest interest to know the part that such reversible enzyme action may play in the synthesis of proteins, fats, carbohydrates, and other substances in the animal body.

OXIDATION AND REDUCTION SYSTEMS

Most if not all of the energy of living matter is derived from oxidative processes. Oxidation involves the loss of negative electrons from the substance oxidized, these electrons passing to the oxidizing agent which is simultaneously reduced. Thus the reaction of ferric chloride with potassium iodide involves a transfer of electrons from the iodide ions to the ferric ions so that the iodide is said to be oxidized and the iron reduced. Biological oxidations involve the same transfer of electrons.

$$Fe^{+++} + 3Cl^- + K^+ + I^- \rightleftarrows Fe^{++} + 3Cl^- + K^+ + I$$

In many oxidations the products of the reaction possess less energy than the reacting substances, and in these cases energy is liberated as heat or in some other form.

Many oxidations are coupled with the formation of esters of phosphoric acid such as adenosinetriphosphate. Inorganic phosphate is used to form these esters. The esters which are formed primarily are very labile, and there is a considerable free-energy decrease (about 10,000 calories) when they are hydrolyzed. Such an ester is adenosinetriphosphate, which contains two such labile phosphoric acid groups. These labile phosphoric acid residues are connected in a manner similar to that found in pyrophosphoric acid and the bond is sometimes called a high-energy phosphate bond. The formation of such compounds is a matter of considerable significance. It suggests how a cell might make use of the energy of oxidation. For example, in the presence of hexokinase from yeast or muscle, the following reaction occurs:

$$\text{adenosinetriphosphate} + \text{glucose} \rightarrow \text{adenosinediphosphate} + \text{glucose-6-phosphate}$$

Thus glucose is changed into a form in which it can readily enter into the intermediate reactions of carbohydrate metabolism. One might ask why phosphatase could not do the same thing with glucose. Phosphatase catalyzes the following reaction:

$$\text{glucose-6-phosphate} \rightarrow \text{glucose} + \text{phosphate}$$

It is commonly considered that all enzyme reactions are reversible. However, thermodynamic considerations suggest that, although this reaction may be reversible, the equilibrium would be so far to the right that the reverse reaction would be physiologically insignificant. Thus there would be very little glucose-6-phosphate formed. On the other hand, in the reaction cited with adenosinetriphosphate, the equilibrium position is far to the side of glucose-6-phosphate and adenosinediphosphate. In this way it is thought that the cell can use at least part of the energy produced by oxidation reactions to form materials which are useful to the cell.

Oxidation of Hydrogen. The first steps in the oxidation of fats, carbohydrates and proteins are dehydrogenations, brought about by enzymes known as dehydrogenases. About 40 dehydrogenases are known. In many instances the fats, carbohydrates, and proteins are not dehydrogenated as such, but are first broken down by hydrolysis or desmolysis to simpler compounds, i.e. diphosphoglyceric aldehyde, glycerophosphate, choline, amino acids, etc.

The individual dehydrogenases are frequently composed of a specific protein loosely united with coenzyme I or coenzyme II. Coenzyme I is also known as diphosphopyridine nucleotide, or DPN. Coenzyme II is triphosphopyridine nucleotide, or TPN.

The hydrogen that is split off unites with these coenzymes, forming DPNH and TPNH. The hydrogen may be next taken up by TPN–cytochrome C reductase, or by DPN–cytochrome C reductase:

TPNH + TPN–cytochrome C reductase ⇄ TPN
+ reduced TPN–cytochrome C reductase

This enzyme next reacts with cytochrome C:

reduced TPN–cytochrome C reductase + cytochrome C
⇄ TPN–cytochrome C reductase + reduced cytochrome C + H^+

Finally, in the presence of atmospheric oxygen and cytochrome oxidase, the oxidation is completed:

$$\text{reduced cytochrome C} + 2H^+ + \tfrac{1}{2}O_2 = \text{cytochrome C} + H_2O$$

Some of the hydrogen present in substrates enters the Krebs cycle and is split off at one stage or another. One of these reactions involves succinic acid, which is oxidized by the succinic oxidase system. Here the initial reaction is:

succinic acid ⇄ fumaric acid + 2H
(succinic dehydrogenase)

The route traveled by the hydrogen is probably as follows:

succinic acid → cytochrome B → cytochrome C
→ cytochrome A → cytochrome oxidase

If some easily reduced dye, such as methylene blue, is present it will be reduced to leucomethylene blue in the presence of succinic acid:

succinic acid + Mb ⇄ fumaric acid + MbH_2
(succinic dehydrogenase)

When leucomethylene blue comes in contact with oxygen it is reoxidized, forming methylene blue and hydrogen peroxide:

$$MbH_2 + O_2 = Mb + H_2O_2$$

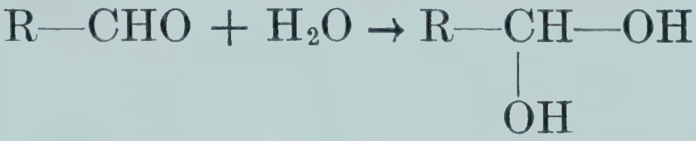
A number of yellow enzymes (old yellow enzyme, xanthine oxidase, glucose oxidase of molds, D-amino acid oxidase) in the reduced state can react with gaseous oxygen to form hydrogen peroxide. In living cells this hydrogen peroxide is very rapidly decomposed by the enzyme catalase. This reaction is:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Catalase is a heme-protein found in all living cells, with the exception of certain bacteria. Its function appears to be the removal of the hydrogen peroxide, which is toxic. Keilin and Hartree have observed that catalase can utilize hydrogen peroxide to oxidize methyl and ethyl alcohols to the corresponding aldehydes. On account of this reaction Theorell has sugested that catalase is an important peroxidizing enzyme. However, this ypothesis has little to support it at the present time.

The hydrogen peroxide may also be removed by peroxidase. This nzyme, in the presence of H_2O_2, oxidizes various phenolic compounds nd amines, such as pyrogallol, guaiacol, hydroquinone, tyrosine, adrenalne, etc. Peroxidase is a heme-protein; in fact, even hemin itself has a ery slight peroxidase action. Peroxidase, like other heme-containing nzymes, is inhibited by HCN, H_2S, and sodium azide.

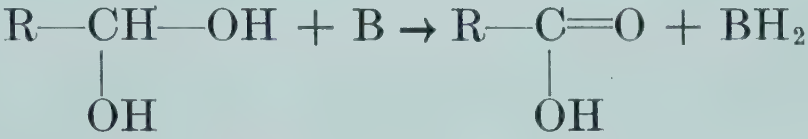
Oxidation by activation and transfer of hydrogen also occurs in the xidation of aldehydes. First of all, the aldehyde is hydrated:



$$R{-}CHO + H_2O \rightarrow R{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}OH$$

Aldehyde **Hydrated aldehyde**

The hydrated aldehyde is then oxidized:



$$R{-}\underset{\displaystyle OH}{\underset{|}{CH}}{-}OH + B \rightarrow R{-}\underset{\displaystyle OH}{\underset{|}{C}}{=}O + BH_2$$

Hydrated aldehyde **Acid**

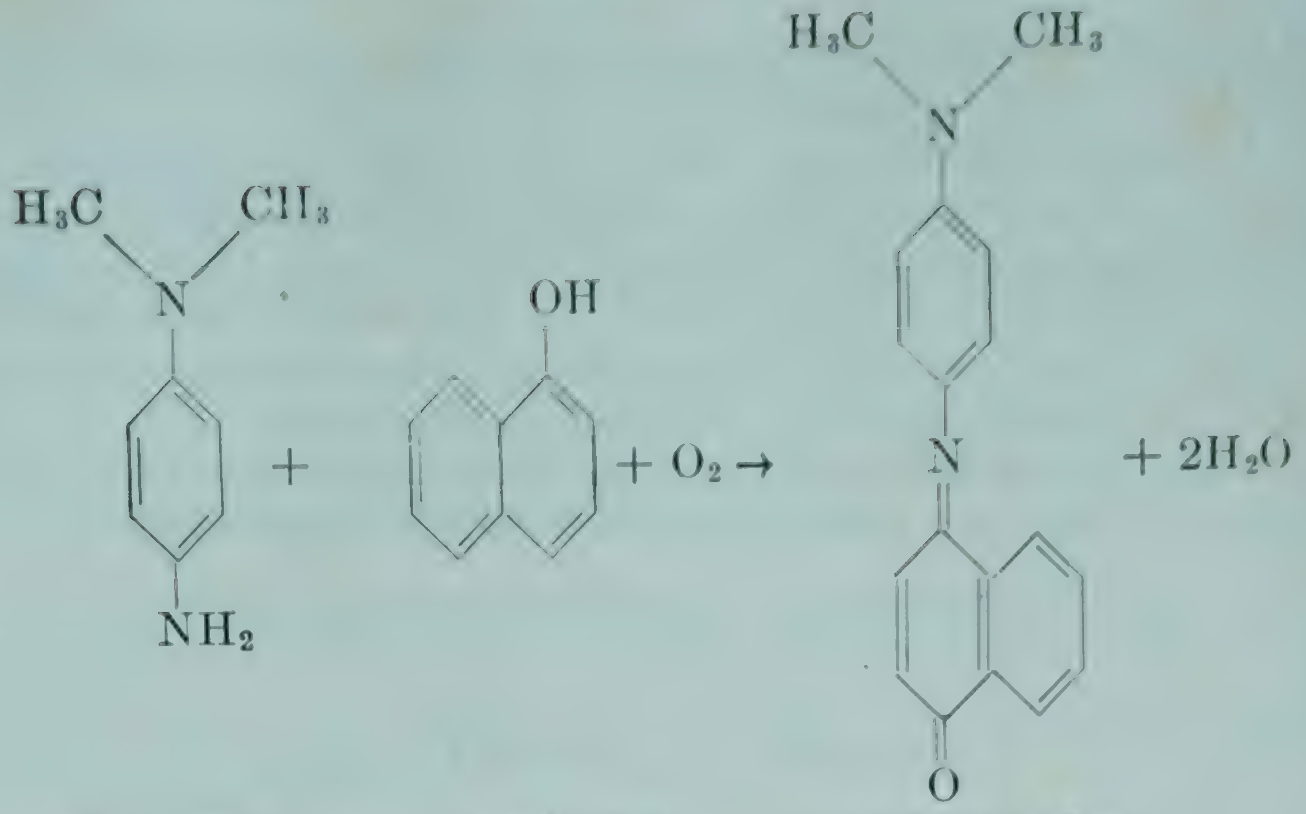
This type of oxidation occurs in the oxidation of acetaldehyde to acetic acid by aldehyde dehydrogenase. Similarly alcohol dehydrogenase nediates the oxidation of alcohols to aldehydes; as, for example, vitamin A to retinene.

Cytochromes. By use of the spectroscope, Keilin was able to demonstrate in living cells the presence of three closely related substances which he called cytochromes A, B, and C. Cytochromes appear to be present in all living cells except a few bacteria. The cytochromes are heme-protein compounds. The absorption bands of the reduced cytochromes may be easily observed by looking at a yeast suspension with a spectroscope. If a stream of air is bubbled through the suspension, the cytochromes are oxidized and the bands disappear. In animal tissues the cytochromes commonly exist predominantly in the oxidized form.

Oxidation by Activation of Oxygen. Warburg found in living cells a heme-containing compound which has the property of activating oxygen and which he called the respiratory enzyme. The activity of the enzyme is dependent upon the iron present, since HCN, in small amounts, and carbon monoxide inhibit the action, apparently through combination with the metal. The inhibition by carbon monoxide can be obtained only n the dark. Warburg's respiratory enzyme is apparently identical with the enzyme formerly called indophenol oxidase and now known as cytochrome oxidase.

Cytochrome oxidase, in the presence of molecular oxygen, oxidizes reduced cytochrome C, as has already been described.

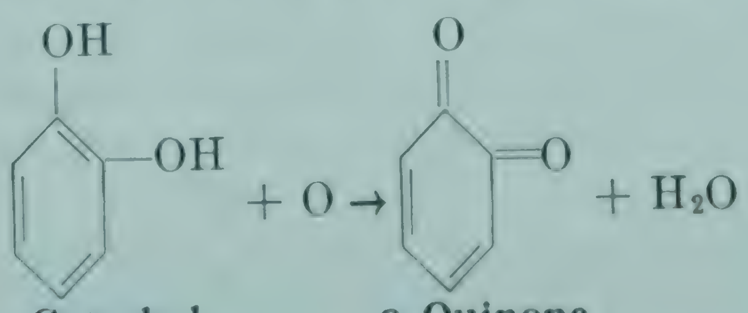
Cytochrome C is reduced by such substrates as hydroquinone and by the Nadi reagent, a mixture of dimethyl *p*-phenylenediamine and α-naphthol. Here the reaction is:



Dimethyl-*p*-phenylenediamine **α-Naphthol** **Indophenol blue**

Yellow Enzymes. TPN–cytochrome C reductase and DPN–cytochrome C reductase are yellow enzymes. These enzymes contain riboflavin (vitamin B_2 or G) in their prosthetic groups. The prosthetic group of cytochrome C reductase consists of riboflavin phosphate (isoalloxazine-D-ribose-phosphate) which is sometimes called a mononucleotide. There are other yellow enzymes which contain such mononucleotides as prosthetic groups. An example is the old yellow enzyme of Warburg. Other yellow enzymes contain prosthetic groups composed of riboflavin-phosphate-phosphate-D-ribose-adenine. Such a compound is called a dinucleotide. Examples of yellow enzymes which contain isoalloxazine-adenine dinucleotide prosthetic groups are diaphorase, the Haas yellow enzyme, xanthine oxidase, and D-amino acid oxidase. These yellow enzymes function in hydrogen transport as outlined above. It is the prosthetic group of these enzymes which is oxidized and reduced.

Copper Enzymes. There are various enzymes which contain copper. These are all inhibited by HCN in much the same way as the iron-containing enzymes cytochrome oxidase, catalase, and peroxidase. One of these copper enzymes is tyrosinase. This enzyme is apparently identical with monophenoloxidase and polyphenoloxidase. This enzyme oxidizes various phenolic compounds such as phenol, catechol, cresols, tyrosine, pyrogallol, and dopa (3,4-dihydroxyphenylalanine). For example:

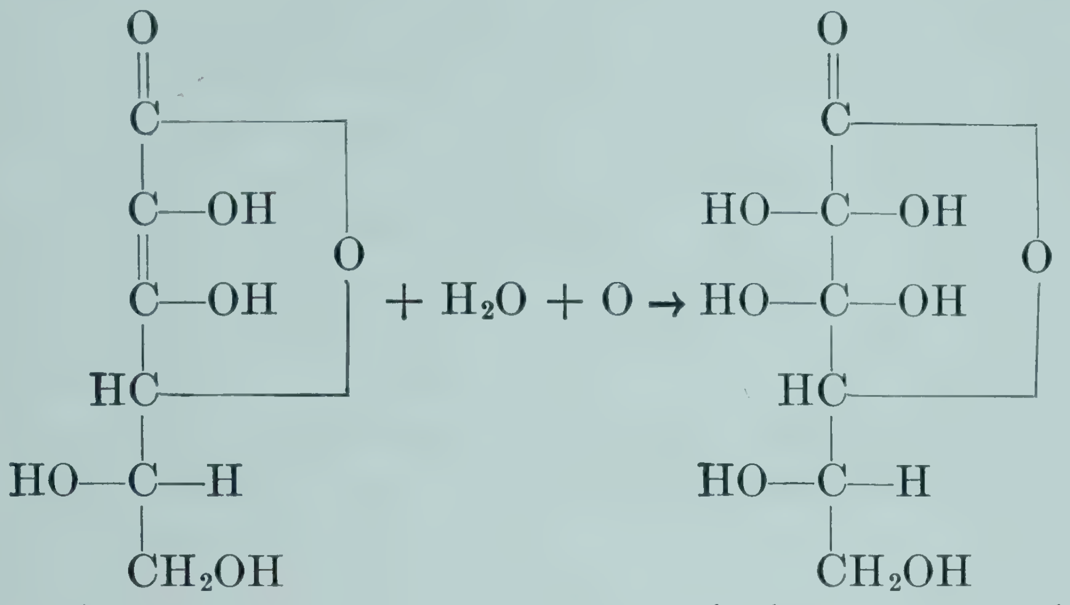


Catechol **o-Quinone**

Water, not hydrogen peroxide, is always formed as a result of oxidation by this enzyme, and gaseous oxygen is used as the hydrogen acceptor.

Tyrosinase is also inhibited by H_2S and by CO. The inhibition by carbon monoxide is not influenced by light. Tyrosinase is commonly found in plant tissues, bacteria, and fungi; it also occurs in animals. Tyrosinase is very similar to laccase, which is also a copper-containing enzyme. Laccase is found in various plant tissues and differs from tyrosinase in that it does not oxidize tyrosine or *p*-cresol.

Ascorbic acid oxidase is another plant copper-containing enzyme. It catalyzes the oxidation of L-ascorbic acid (vitamin C) in the presence of oxygen:

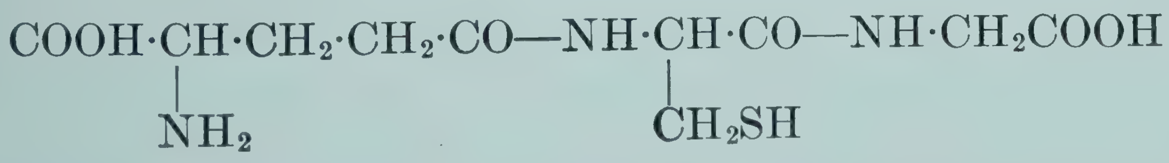


L-Ascorbic acid **Dehydroascorbic acid (hydrated)**

The dehydroascorbic acid may be reduced by hydrogen sulfide.

It has been proposed by various workers that one or more of these copper-containing enzymes function in plant tissues in much the same way as does cytochrome oxidase in animal tissues. The matter is as yet unsettled.

Sulfhydryl Group in Oxidations. Practically all tissues give a purple color with sodium nitroprusside and ammonia. This indicates the presence of the sulfhydryl group R—SH. Such a group is found in the amino acid cysteine $HOOC{\cdot}CH(NH_2){\cdot}CH_2SH$. Cysteine is not, however, found to any extent in the tissues as such but is found in appreciable amounts in the form of glutathione, a tripeptide of glycine, cysteine, and glutamic acid, which has the following structure:



$$\underset{\displaystyle NH_2}{COOH{\cdot}\underset{|}{CH}}{\cdot}CH_2{\cdot}CH_2{\cdot}CO{-}NH{\cdot}\underset{\displaystyle \overset{|}{CH_2SH}}{CH}{\cdot}CO{-}NH{\cdot}CH_2COOH$$

On oxidation cysteine may be converted into the disulfide form, or cystine: $R{-}SH + HS{-}R + O \rightarrow R{-}S{-}S{-}R + H_2O$; and the cystine may be reduced to cysteine: $R{-}S{-}S{-}R + 2H \rightarrow 2R{-}SH$. Glutathione may also be oxidized to the disulfide form $2G{-}SH \rightarrow G{-}S{-}S{-}G$, this reaction also being reversible. The widespread occurrence of glutathione and the amounts found in cells indicate its importance, but its mode of action is not yet clear. Glutathione has been synthesized both by methods of organic chemistry and by the use of enzymes.

New Coenzymes. In recent years the list of coenzymes has increased considerably. Thus, we have uridine diphosphoglucose necessary for the conversion of galactose-1-phosphate to glucose-1-phosphate by the enzyme known as phosphogalactose isomerase. Coenzyme III, found in yeast, is required for the oxidation of L-cysteine sulfinic acid to cysteic acid.

A yet more interesting coenzyme is *coenzyme A*, containing pantothenic acid (q.v., Chapter 35), adenylic acid, and mercaptoethanolamine. Coenzyme A functions in the acetylation of aromatic amines by pigeon liver homogenates in the presence of acetic acid and ATP. Here the intermediate product is acetyl coenzyme A. Coenzyme A is required for the acetylation of choline by nervous tissue. Aged extracts of autolyzed pigeon liver convert oxalacetic acid to citric acid if coenzyme A, ATP, magnesium ions and acetate are present. Coenzyme A is necessary for the catalytic transfer of acetyl-bound phosphate, as well as for the arsenolysis of acetyl phosphate by the phosphotransacetylase of *Clostridium kluyveri*. In this process coenzyme A accepts an acetyl group from pyruvic acid and forms acetyl phosphate. The enzyme present is phosphotransacetylase, which occurs neither in animals nor in yeast. Bacterial phosphotransacetylase together with an enzyme from pigeon liver can employ synthetic acetyl phosphate to synthesize acetoacetic acid.

Coenzyme A is thought to be involved in the synthesis of phospholipides. Here ATP supplies the energy. It was found that yeast extracts convert coenzyme A to a pyrophosphate in the presence of ATP. This compound can react with acetate to give rise to acetyl coenzyme A and pyrophosphate.

6-Thioctic acid (lipoic acid, or 6,8-dithio-n-octanoic acid) has been isolated in crystalline form from liver and from yeast. This substance is necessary for the growth of the protozoan *Tetrahymena gelii* and is able to replace acetate needed for the growth of *Lactobacillus casei*. It appears that lipoic acid is necessary for the oxidation and dismutation of pyruvic acid by *Streptococcus fecalis*, and that the formation of acetyl coenzyme A and succinyl coenzyme A requires the presence of both thiamine pyrophosphate and lipoic acid. Thioctic acid is discussed further in Chapter 35.

EXPERIMENTS ON ENZYMES[5]

ROLE OF IRON IN OXIDATIONS

1. *Catalytic Decomposition of Hydrogen Peroxide by Metals.* Transfer 5-ml. portions of hydrogen peroxide to each of 4 test tubes. To one add a very small amount of finely divided metallic platinum,[6] to the second a small amount of powdered magnetite (Fe_3O_4), to the third a similar amount of ordinary ferric oxide or hematite (Fe_2O_3), and to the fourth a small amount of magnetite and a few drops of 1 per cent NaCN (*Poison!*). Note any evolution of gas. After

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[5] If it is deemed advisable by the instructor to give all the practical work upon enzymes at this point in the course, additional experiments will be found in the chapters on digestion.

[6] Use a solution of colloidal platinum (see p. 10) or a powder prepared by dipping filter paper in platinic chloride solution and igniting in a crucible. Welo and Baudisch have shown that magnetite produces active oxygen even when heated to 330° C., although it then has the same composition (Fe_2O_3) as it has when heated still further to 550° C.; but the latter product sets free molecular, not active, oxygen. Ferrous hydroxide also catalyzes a rapid decomposition of peroxide.

a few minutes add a few drops of an alcoholic solution of guaiac. This is oxidized to a blue color by active oxygen. Which tubes show this reaction? Cyanide paralyzes cell respiration, apparently by inactivating the iron which is an essential part of the system.

EXPERIMENTS ON PLANT OXIDASES

1. *Demonstration of Potato Oxidases.* **It is convenient to combine the study of potato oxidases with a study of the composition of the potato. This throws light on the value of the potato as a food. It also gives information as to the composition of a typical vegetable cell.[7]**

(a) PREPARATION OF POTATO EXTRACTS. **Wash and peel a medium-sized potato. Grate rapidly and transfer the gratings at once to a piece of cheesecloth which is suspended in a beaker containing about 200 ml. of distilled water. Work gently with the hand to get out as much of the starch as possible. Keep this extract (water extract No. 1). Make a second extraction using 200 ml. of distilled water (water extract No. 2). Make a third extraction, and if this does not contain an appreciable amount of starch, discard it. Work the pulp until it is practically starch-free.**

(b) TESTS ON PULP. **Work a portion of the pulp very thoroughly with water until it is practically free from starch as indicated by the iodine test. Test for protein, using Millon's test, and for carbohydrate by the Molisch test.**

(c) TESTS ON WATER EXTRACT NO. 1. **Pour off the supernatant liquid from the extract when the starch has settled out. Filter. Test for reducing sugars by Benedict's test. Test for protein. Boil 20 ml. of the extract and filter. Test the filtrate for inorganic chlorides, sulfates, and phosphates. Indicate in your notebook what information you have obtained as to the food value of the potato.**

(d) SEPARATION OF STARCH. **Combine the starch obtained from extracts No. 1 and No. 2. Wash by decantation with distilled water. Drain off the water and turn the beaker upside down so that the starch will drain; otherwise molds may develop.**

(e) EXPERIMENTS ON POTATO OXIDASE. **Into each of a series of 5 clean test tubes introduce 5 ml. of potato extract (extract No. 2 filtered). (If the extract is kept over, it must be preserved with toluene.) Introduce other reagents according to the following series: (1) Potato extract + 10 drops of 1 per cent phenol,[8] (2) potato extract + 10 drops of 1 per cent catechol, (3) potato extract + 10 drops of guaiac solution, (4) potato extract + 10 drops of pyrogallol solution, and (5) potato extract + 5 drops of α-naphthol solution + 5 drops of *p*-phenylenediamine hydrochloride solution. This combination of α-naphthol and *p*-phenylenediamine is known as the Nadi reagent.**

Mix the contents of the tubes by shaking. Watch for any color changes. If necessary let stand until the next laboratory period (add toluene), and examine again. In this experiment the phenol C_6H_5OH, catechol $C_6H_4(OH)_2$, and pyrogallol $C_6H_3(OH)_3$ are oxidized with the production of brown-colored compounds. The guaiaconic acid in the guaiac is oxidized to guaiac blue. In the last tube we have the production of indophenol from the α-naphthol and phenylenediamine under the influence of oxidase.

Two oxidases appear to be active in these tests. Monophenoloxidase (also called polyphenoloxidase and tyrosinase) is responsible for the oxidation of the phenol to catechol, then to *o*-quinone, and finally with condensation reactions to brown compounds of uncertain composition.

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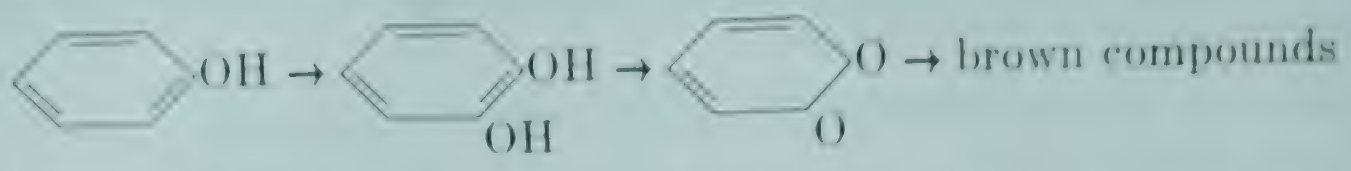
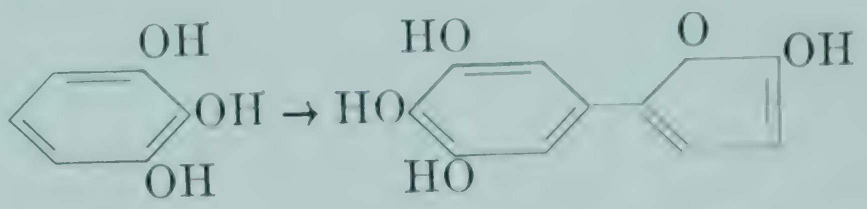
[7] This experiment is based upon the laboratory directions of Dr. William H. Welker.

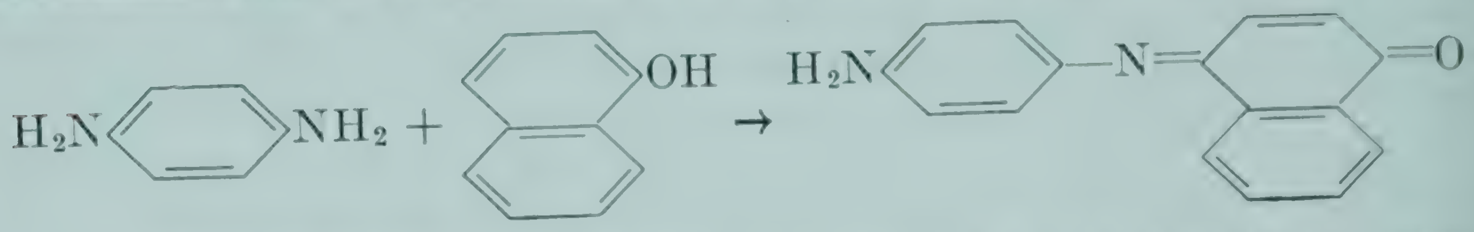
[8] The phenol, catechol (pyrocatechol), *p*-phenylenediamine hydrochloride, and pyrogallol are 1 per cent aqueous solutions. The α-naphthol is a 1 per cent solution in 95 per cent alcohol. For the guaiac solution dissolve 0.5 g. of gum guaiac in 30 ml. of 95 per cent alcohol.



Polyphenoloxidase also acts upon catechol to form *o*-quinone and then the brown compounds just mentioned. It also acts upon pyrogallol forming purpurogallin.



Cytochrome oxidase in the presence of cytochrome C oxidizes the *p*-phenylenediamine and in the presence of α-naphthol there is formed indophenol.



Peroxidase in the presence of hydrogen peroxide also gives this reaction. At least part of the color obtained in the above test is due to the action of peroxidase since the color develops more slowly if catalase is added to the potato oxidase preparation.

The oxidation of the guaiaconic acid of the guaiac to guaiac blue is apparently due to the action of the *o*-quinones formed as indicated above, on the guaiac, rather than any direct action of the enzymes on the guaiac itself except in the case of the slight peroxidase action due to the presence of some peroxide. Peroxidase appears to oxidize the guaiac directly.

2. *Experiments on Potato Peroxidase.* Prepare a series of 5 tubes containing 5-ml. portions of potato extract and 10 drops of oxidase reagents as in the preceding experiment. Prepare still another series, but use potato extract previously boiled for 5 minutes. Then to each tube add 10 drops of 3 per cent hydrogen peroxide solution. Note whether oxidation takes place more rapidly than in Exp. 3 where no hydrogen peroxide is used. Is the peroxidase destroyed by boiling?

The potato has a greater peroxidase than phenoloxidase activity, which accounts for the more rapid action in the presence of H_2O_2.

3. *Resistance of Oxidase and Peroxidase to Heat.* Into each of 3 test tubes introduce 5 ml. of potato extract. Put in a water bath at 70° for 10 minutes. To the first tube add 2 drops of 1 per cent catechol solution and then to each 10 drops of guaiac solution, and to the third tube only, 10 drops of hydrogen peroxide solution. The appearance of a blue color in the presence but not in the absence of hydrogen peroxide indicates that peroxidase is not destroyed at 70° but that the phenol oxidase is not stable at this temperature. How would you prepare a solution containing peroxidase but not oxidase? The

fact that the addition of catechol does not suffice to bring back the direct oxidase action indicates that it is not the catechol compounds but the oxidase that is destroyed at this temperature.

4. *Role of Catechol Compounds in Oxidase System.* Into each of 2 clean test tubes introduce 5 ml. of potato extract. To one, add 1 drop of 1 per cent catechol solution. Let stand for 5 minutes. Then to each tube add 10 drops of guaiac solution. Let stand and note any color change.

Most potatoes contain relatively little of the catechol compounds. The addition of catechol therefore hastens the oxidation of the guaiac, since the oxidation of the guaiac is secondary to the formation of *o*-quinones from catechol compounds, the *o*-quinones being capable of directly oxidizing guaiac.

5. *Preparation of Peroxidase.* Scrapings of horseradish may be extracted with alcohol and dried. On extraction with water these dried scrapings give a peroxidase solution free from oxidase. By a more complicated process a preparation showing about 1,000 times the activity of the original material has been obtained.

6. *Determination of Peroxidase.* Into a 250-ml. flask introduce 100 ml. of a saturated solution of leucomalachite green,[9] 2 ml. of 0.166 N sodium acetate saturated with toluene, 1 ml. of hydrogen peroxide,[10] and from 0.0025 to 0.05 unit of peroxidase in not more than 5 ml. of solution. All should be brought to 20° C. before mixing and kept at that temperature. Measure 10 ml. of N H_2SO_4 into a small flask, and add all at once at the end of 5 minutes to the digestion mixture to stop the action. Rinse the small flask with 5 ml. of water. After 15 to 30 seconds, neutralize with a little more than an equivalent amount of NaOH solution. Shake vigorously to eliminate bubbles of CO_2. Compare with a standard malachite green solution containing 10 mg. per liter, and set at 5 or 10 mm. The standard is made up in 0.05 N acetic acid and preserved with toluene. Yellow artificial light is better than daylight for the comparison. Under the given conditions one unit of peroxidase forms 53 mg. of malachite green.

7. *Demonstration of Catalase.* Mix about 1 g. ground liver with 3 ml. water and add a few ml. 3 per cent hydrogen peroxide. Test the gas evolved for oxygen, using a glowing match.

EXPERIMENTS ON ANIMAL OXIDASES

1. *Schardinger Reaction.* Place 5 ml. of milk in each of 3 test tubes. Heat one to boiling and cool. To each tube add 1 ml. of 0.02 per cent methylene blue solution. To tubes 1 and 2 add 1 ml. of 0.4 per cent formaldehyde solution. Mix by gentle rotation, add 1 to 2 ml. of paraffin oil, and put in a water bath at about 40° C. The milk in tube 2 should gradually decolorize. The

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[9] The dye should be recrystallized twice from alcohol, once from petroleum ether, and again from alcohol. Saturate titrated 0.05 N acetic acid with the dye at 20° C. The solution freed from air under a vacuum will keep for months. If precipitation occurs, warm to redissolve.

[10] Dilute 30 per cent hydrogen peroxide (reagent grade) 100 times. Titrate an aliquot with $KMnO_4$ after adding sulfuric acid and dilute further so that each ml. contains 0.25 mg. H_2O_2.

reaction is an example of anaerobic oxidation in the presence of a hydrogen acceptor (methylene blue) and may be written thus:

$$CH_2O + H_2O \rightarrow CH_2(OH)_2 + M \rightarrow HCOOH + M{\cdot}H_2$$

This reaction is given more slowly by milk which has been heated and more rapidly by milk which has a high bacterial count.

2. Study of Tissue Oxidations by the Methylene Blue Method of Thunberg and Ahlgren: Principle. Finely divided tissue is suspended in a solution containing methylene blue, phosphate solution to regulate the acidity, and the substance whose action it is desired to determine. The tube is evacuated, placed in a water bath, and the time required for the methylene blue to be decolorized is determined. This is a measure of the rate of oxidation in the mixture. The nature of the substances capable of being oxidized by the tissue can be determined and the influences governing the oxidation process studied.

Procedure. The tube[11] most convenient for use in this experiment is illustrated in Fig. 77. Into each of 3 tubes introduce 0.9 ml. of a mixture of 8 ml. of methylene blue 1:2000 and 6 ml. of a phosphate buffer of pH 7. Then add 0.1 ml. of water to the first tube, of 0.1 M potassium succinate to the second, and of 0.1 M potassium glycerophosphate to the third. Then add 0.2 g. of finely divided washed rabbit muscle[12] to each. Evacuate each tube for 2 or 3 minutes with a strong water-jet filter pump.[13] Place the tubes in a constant-temperature water bath at 35° C. Observe from time to time and note when each tube just loses its last trace of blue color. Record the number of minutes required in each case. Do the succinate and glycerophosphate solutions decolorize first? What does this indicate as to the oxidation of these substances by muscle tissue?

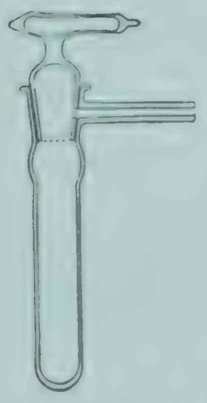

FIG. 77. VACUUM TUBE. (THUNBERG.)

3. Nitroprusside Reaction for Glutathione. Rub up in a mortar a small piece of fresh liver or kidney tissue, a little sand, and a few ml. of saturated

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[11] The tube is made of hard, colorless glass and is of about 10 ml. capacity. The stopper is greased with a mixture of: rubber 1 part, petroleum jelly 2 parts, and heavy liquid petrolatum 1 part. Tubes may be obtained from the Scientific Glass Apparatus Co., Fisher Scientific Co., and others.

Solutions required are: Stock solution of methylene blue, 1 g. in 500 ml. of water. Phosphate buffer solution of pH 7.

[12] The muscles of frogs and other animals may also be used. The animals should be killed by a blow and not by anesthesia. One or more g. of muscle tissue freed as much as possible from ligaments, fat, and blood are cut up on a watch glass with a scissors of the Cooper type for 3 to 5 minutes. A drop or two of water may be added to assist cutting. The tissue may then be washed if desired by shaking with an excess of water, rubbing in a mortar, straining through silk, and drying with filter paper. Keep in a covered beaker placed in a dish containing pieces of ice. The divided tissue is best used as soon as possible but may generally be kept for an hour at 20° C. Uncut tissue may be kept somewhat longer. In weighing the tissue a microbalance weighing up to 500 mg. is convenient, a small celluloid shell being attached to hold the material. A glass rod with a spoon-shaped tip is used to introduce the tissue into the tube.

[13] There may be some foaming during the evacuation, which should be continued until the solution boils when warmed with the hand. Foaming may be reduced by rotating the tube in a horizontal position so as to distribute the material on the sides of the tube or by first putting the tubes in cold water. After evacuation, to get all material into the bottom of the tubes they may be rotated for a few seconds in a hand centrifuge. It is also well after evacuation and closing the stopper to remove the tube from the side tube while holding both under water. The side tube is filled with water so that leakage of air is less probable.

ammonium sulfate solution. Add 5 or 6 drops of 2 per cent sodium nitroprusside and 2 ml. of concentrated ammonium hydroxide solution. The mixture turns purple.

4. *Estimation of Glutathione in Tissue.* See Chapter 23.

5. *Preparation of Glutathione:*[14] *Procedure.* Add 20 ml. of concentrated H_2SO_4 slowly to 100 ml. of 89 per cent alcohol, keeping the mixture cool. When cold add 80 ml. of ether. Pour on to 2,000 g. of compressed bakers' yeast, coarsely crumbled in a large jar. Stir for a few minutes until homogeneous. Pour on to 2 large Buchner funnels, the papers being covered with thin layers of kieselguhr. After 4 hours, filtration may nearly stop. If so, pour off the fluid upper layer on to another filter, and suck dry for a few hours more. Do not use too high a vacuum at first or the mixture will froth as the ether boils off.

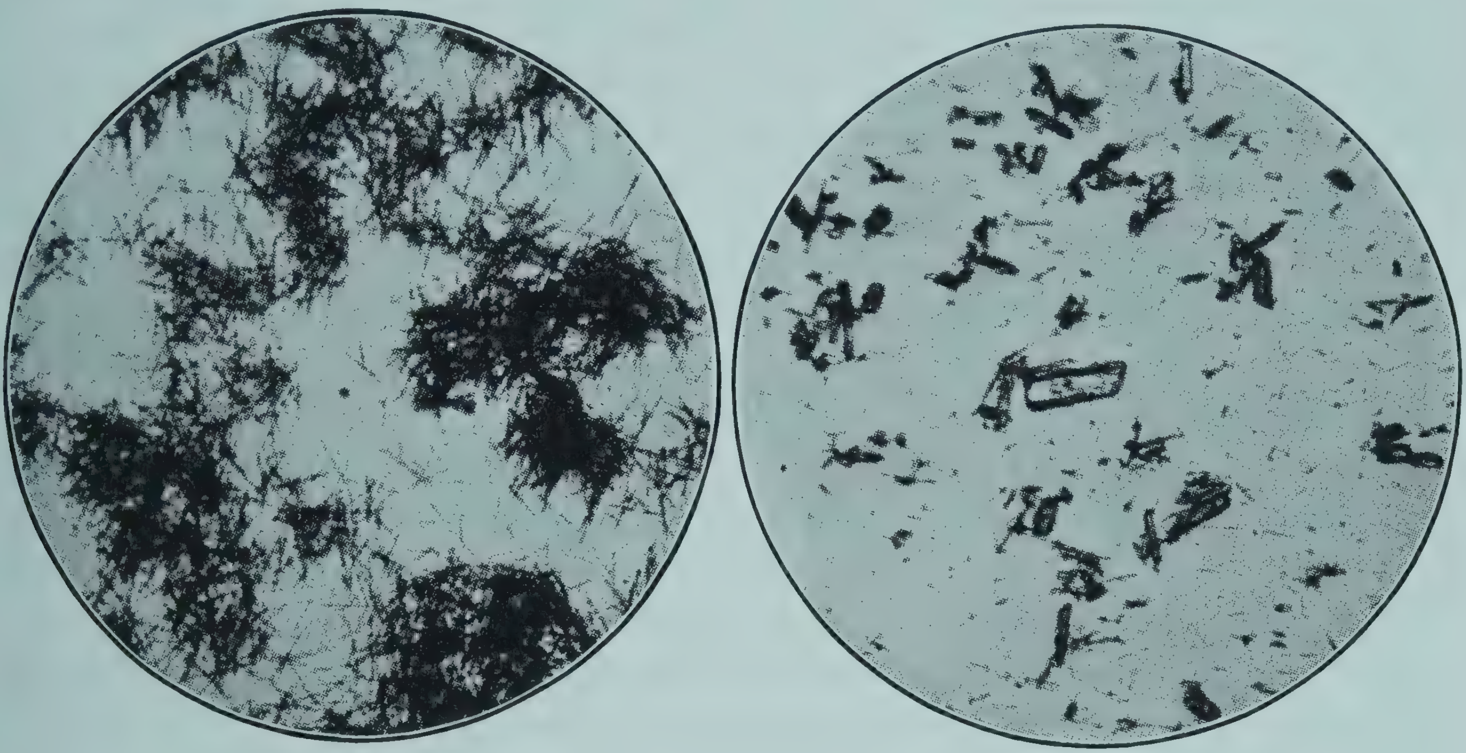

FIG. 78. CRYSTALS OF GLUTATHIONE.
(*Left*) First crystallization. (*Right*) Recrystallized.
Courtesy, Kendall, McKenzie, and Mason: *J. Biol. Chem.*, **84**, 657 (1929).

The combined filtrates (about 1100 ml.) are titrated with NaOH, using Congo red as an indicator (the filtrate is generally just acid to Congo red), and the amount of 20 per cent H_2SO_4 necessary to bring the filtrate to 0.5 N added.

Prepare about 1 g. of cuprous oxide by boiling ordinary Fehling's solution with an excess of glucose cautiously added. It should be bright red. Wash by decantation on a filter. It may be kept in a dry state. Rub up and suspend in about 30 ml. of water. With thorough stirring add 2-ml. portions of suspension at a time to the yeast extract previously carefully warmed on a water bath with stirring to 50° C. When about half of the oxide has been added, allow the salt to settle, and pour the mother liquor off for further additions of oxide. At a later stage the addition of cuprous oxide will cause the re-solution of some of the cuprous-glutathione compound already formed. When this occurs, reprecipitate by blowing air through the solution for about an hour. The precipitate redissolves on very prolonged aeration. Although

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[14] For a procedure involving the use of a supercentrifuge see Kendall, Mason, and McKenzie: *J. Biol. Chem.*, **87**, 55 (1930); **88**, 409 (1930).

flocculent and amorphous, this compound has the normal copper content (17 per cent) and gives crystalline glutathione on decomposition.

Wash the cuprous salt with 0.5 N H_2SO_4 on the centrifuge and then with water until the washings are free from sulfate. If a centrifuge is used, about 10 washings are required. Or use a device prepared with two Jena sintered glass filters (grade 4) of suitable size. Put the cuprous salt in one and place the other upside down on top, a water-tight joint being made with a piece of wide rubber tubing. Connect the lower funnel to a reservoir of distilled water about a meter above it, and attach the upper one to a flask and filter pump. Wash until the filtrate remains free from sulfate after shaking the filtration apparatus.

Suspend the precipitate in four to five times its bulk of distilled water, and decompose with well-washed H_2S. Filter. Free from H_2S by a stream of hydrogen. Evaporate to a small bulk in a vacuum desiccator at room temperature. If the volume of filtrate is too large for this and distillation in vacuum is necessary, use a good pump and distil at 25° C. If the mixture does not crystallize spontaneously before reaching a sirupy consistency, rub with a glass rod to start crystallization. Yield, about 1.5 g. For the crystalline form of glutathione, see Fig. 78.

PREPARATION AND PURIFICATION OF ENZYMES[15]

Purified enzyme preparations may be made from digestive secretions containing the enzyme. More often, however, the source is an animal or plant tissue. To obtain the enzyme in concentrated form it must be freed as far as possible from the mixture of substances making up the cell.

In the case of animal tissues the material should be immediately refrigerated and used as soon as possible. It may be cut up with a scissors or run through a meat-chopper and then rubbed in a mortar with quartz sand or ground glass. Freezing with liquid air or carbon dioxide will often aid comminution and check fermentative processes. The material may also in some cases be dehydrated by drying in a current of warm air or by dehydrating with acetone or alcohol followed by treatment with ether to remove fat, and then grinding very fine in a ball mill.

The comminuted tissue may then be extracted by use of a suitable solvent (e.g., water, salt solutions, glycerol, or solutions of definite pH) which will extract the enzyme with as little other material as possible. The mixture may then be filtered or centrifuged. Since enzymes are proteins, the greatest difficulties in enzyme purification are connected with the removal of protein contaminations. Methods used in the purification of proteins are therefore useful (see Chapter 6).

Just as with proteins, the exact procedure will vary in each case. The procedures generally required include dialysis, precipitation by means of salts or by liquids such as alcohol or acetone, and selective adsorption and elution. Dialysis, employing most commonly the collodion bag, removes soluble diffusible substances including the salts that may be used in precipitation. Electrodialysis may also be employed. By means of fractional precipitation with salts such as ammonium sulfate, separation from many protein materials may be accomplished. In some cases alcohol may inactivate the

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[15] This subject has been reviewed by Schwimmer and Pardee: *Advances in Enzymol.*, **14**, 375 (1953).

enzyme. Among the most useful adsorbents are different types of aluminum hydroxide. For example, by choosing a suitable form of this adsorbent and the proper acidity of solution, separation of the enzymes of the pancreas has been accomplished. Associated with adsorption methods is of course the elution of the adsorbed material from the aluminum hydroxide–enzyme complex by suitable solvents, among which are solutions of alkali phosphates of different reactions or dilute ammonia. For the preparation of the different forms of aluminum hydroxide see footnote 16. Since preparations of pure

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[16] *Preparation of Adsorbents: Alumina A.* Warm 250 g. of $Al_2(SO_4)_3 \cdot 18H_2O$ in 750 ml. of water to 55° C. and pour the solution all at once with the most vigorous mechanical stirring into 2.5 liters of 15 per cent ammonia warmed to 55°. The temperature rises to 58° and is kept between 55° and 60° C. with continued stirring for half an hour. Transfer to a 5-liter flask with a reflux condenser and boil gently for 48 hours. Dilute in a jar with water to 12 liters. Wash 3 times by decantation. Stir the precipitate with 500 ml. of 15 per cent ammonia to decompose the traces of basic sulfate. Wash until for three successive times the wash water no longer comes away clear, and the precipitate becomes a plastic gel from which the wash water can be completely poured.

Alumina B. Precipitate as for *Alumina A.* After the precipitation stir for not more than half an hour. Then dilute at once to 12 liters and wash by decantation as above.

Alumina Cα. Ammonia alum is used. Ammonia used should be standardized by titration and should be measured accurately. Dissolve 22 g. ammonium sulfate in 600 ml. of water. Warm to 63° C. and add 100 ml. of 10 per cent ammonia. Warm quickly to 58°. Add with vigorous mechanical stirring all at once a solution of 76.7 g. of ammonia alum in 150 ml. of water, this solution being previously warmed to 58°. The temperature rises to 61°. Do not let the temperature go below 58° C. Ten minutes after beginning precipitation, separate as rapidly as possible from the mother liquor by centrifuging. Transfer with 1500 ml. of water containing 1.25 g. of NH_3 to a 1500-ml. flask and shake. Centrifuge. Repeat using 2.5 g. of NH_3. Each treatment with ammonia requires about 17 minutes. Then wash three times more, using water only. The last supernatant fluid should remain turbid. The whole procedure from the first precipitation should require about 2¼ hours, and must be expedited as the preparation is unstable.

Alumina Cβ. The α compound changes into the β form on standing a few hours. The flocculent suspension becomes a plastic gel less soluble in acid. The β form changes gradually (10 days to several months) into the γ form.

Alumina Cγ. Precipitate as for the α form. Stir for 15 minutes at 60°. Transfer with 5 liters or more of water to a tall jar and wash by decantation. To the fourth wash water add 80 ml. of 20 per cent NH_3 to decompose the basic sulfate. Wash 12 to 20 times more with water or twice after the supernatant fluid no longer becomes clear. Some months' standing under water is required for complete conversion to the γ form to take place. The precipitate becomes more flocculent and is insoluble in cold dilute or fairly strong HCl.

Alumina D. Dissolve 130 g. of pure commercial aluminum hydroxide with 140 g. of KOH (80 per cent) in 900 ml. of hot water. Dilute to one liter and filter. Dilute to 10 liters. Precipitate by running a gentle stream of carbon dioxide through the solution for two days. Decant and wash 12 times with water containing carbon dioxide and then with distilled water. The final washings remain turbid.

Aluminum Metahydroxide. If one of the above forms of aluminum hydroxide be heated suddenly with ammonia in a sealed tube to 250° C. and kept at that temperature for 8 to 9 hours, there is a complete conversion to the metahydroxide form.

Kaolin. Kaolin is best treated with acid before use. To 500 g. of kaolin add 1500 ml. of pure HCl (sp. gr. 1.18) and warm very slowly so that the mixture begins to boil at the end of the first day and then boil for another day. Wash with water by decantation. Repeat the treatment with acid and washing three times more. Wash with cold water until the washings show practically no acidity but the kaolin itself reacts strongly acid on litmus paper.

Other Adsorbents. Ferric hydroxide, magnesium oxide, and stannic and silicic acids are used. Substrates may also be used as specific adsorbents as tristearin for lipase, casein for trypsin, and coagulated egg albumin for pepsin.

Elution. Solutions of ammonia or of disodium phosphate are most generally useful in freeing adsorbed enzymes from combinations with alumina.

Different enzymes are differently adsorbed. The properties of an enzyme as far as adsorption is concerned may change during purification. In a general way the more finely dispersed gels such as alumina *A*, *B*, and *C* have more adsorptive power than those with less surface such as the microcrystalline *D* and metahydroxide. For numerous applications see Willstätter, *et al.*: *Untersuchungen über Enzyme*, Berlin, Julius Springer, 1928. Also Grassmann: *Ergebnisse Physiol.*, **27**, 407 (1928); Oppenheimer: *Die Fermente und Ihre Wirkungen*, Leipzig, Thieme, Vol. 3, 5th ed., 1929.

crystalline enzymes are of the greatest interest, there follows a description of methods used in the preparation of certain of these. Methods for the preparation of active preparations of vegetable lipase and sucrase are also included since these are more readily obtained from vegetable sources. Other enzyme preparations are discussed in the chapters dealing with digestion.

1. *Preparation of Crystalline Urease.*[17] Dilute 158 ml. of redistilled acetone[18] to 500 ml. at 22° C. (= 31.6 per cent acetone). Pour over 100 g. of Arlco jack bean meal in a beaker. Stir 3 to 4 minutes and filter through a Schleicher and Schüll, No. 595, or Whatman, No. 1, filter. Allow about 150 ml. to filter at room temperature. Complete the filtration in the ice chest at 2° to 2.5° C., over night. Centrifuge off the crystals that form, using cold centrifuge tubes. Examine microscopically (see Fig. 75, p. 308). Drain and stir up with 5 to 10 ml. of cold 31.6 per cent acetone. Centrifuge again. Dissolve the crystals in 15 to 40 ml. of distilled water at room temperature, and free the solution from insoluble matter by centrifuging. The activity of the concentrated solution is not lost very rapidly provided the material is kept in the ice chest.

2. *Preparation of Crystalline Pepsin.* Dissolve 500 g. of Parke Davis pepsin (U.S.P. 1:10,000) in 500 ml. of water and add 500 ml. of N H_2SO_4. Add with stirring 1000 ml. of saturated $MgSO_4$ solution. Filter through fluted paper (S. and S. No. 1450½) and then with suction. Discard the filtrate. The precipitate must not stand at room temperature more than 24 hours.

PRECIPITATE 1. Wash twice with an equal volume of ⅔ saturated $MgSO_4$. Filter with suction. Discard the filtrate.

PRECIPITATE 2. Stir with water to a thick paste and run in M/2 NaOH until complete solution. Great care must be taken to avoid local excess of NaOH. The pH must never rise above 5.0. Add M/2 H_2SO_4 with stirring until a heavy precipitate forms (pH about 3.0). Let stand 3 to 6 hours at 8° C. Filter with suction.

PRECIPITATE 3. Stir with water to a thick paste at 45° C. Add M/2 NaOH carefully until the precipitate dissolves (filter if cloudy and discard the precipitate). Place the beaker containing the precipitate in a vessel containing about 4 liters of water at 45° C., inoculate with pepsin crystals, and allow to cool slowly. Cooling should require 3 to 4 hours and a heavy crystalline precipitate should form at about 30° to 35° C. (see Fig. 76, p. 308). Keep the solution at 20° C. for 24 hours. Filter off the thick crystalline paste with suction.

PRECIPITATE 4. Wash with a small amount of cold water and then with ½ saturated $MgSO_4$ and keep under saturated $MgSO_4$ at 5° C. The filtrate may be treated with M/2 H_2SO_4 to attain a pH of 3.0, the amorphous precipitate filtered off and treated like Precipitate 3.

3. *Preparation of Crystalline Trypsin and Chymotrypsin.* Remove the pancreas from cattle within one hour after slaughter, and immerse in cold N/4 H_2SO_4. Drain off the acid, mince, and suspend for 24 hours in 2 volumes of N/4 H_2SO_4 at 5° C. Strain through gauze. Add solid $(NH_4)_2SO_4$ to 0.4 saturation. Filter. Saturate to 0.7 saturation. Let stand 2 days at 5° C. Dissolve in water and re-fractionate between 0.4 and 0.7 saturation. Dissolve the precipitate from 0.7 saturation in 0.25 saturated $(NH_4)_2SO_4$. Adjust to pH 5.0.

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[17] Sumner: *J. Biol. Chem.* **69**, 435 (1926); **70**, 97 (1926). Sumner and Hand: *J. Biol. Chem.*, **76**, 149 (1928). For the recrystallization of urease see Dounce: *J. Biol. Chem.*, **140**, 307 (1941).

[18] Commercial acetone distilled over fused $CaCl_2$ and soda lime, to remove the water and acid.

Let stand 2 days at 25° C. Filter. Crystals are chymotrypsinogen. (Retain filtrate for trypsin.) Recrystallize chymotrypsinogen in 0.25 saturated $(NH_4)_2SO_4$ at pH 5 about 8 times. Dissolve in N/50 H_2SO_4. Adjust to pH 7.6. Add a trace of trypsin. Let stand 2 days at 5° C. Adjust to pH 4. Salt out in 0.7 saturated $(NH_4)_2SO_4$. Filter. Dissolve in N/100 H_2SO_4. Let stand 24 hours at 25° C. Filter off chymotrypsin crystals.

Take the filtrate from the chymotrypsinogen crystallization for the preparation of trypsin. Adjust to pH 4.0, precipitate in 0.7 saturated $(NH_4)_2SO_4$, and refractionate between 0.4 and 0.7 saturated $(NH_4)_2SO_4$. Filter. Wash the precipitate from 0.7 saturation with sat. $MgSO_4$. Dissolve in 0.4 M borate of pH 9.0. Cool to 5° C. Bring to 0.5 saturation with $MgSO_4$. Let stand 3 days at 5° C. Filter off the crystals of trypsinogen. Wash with 0.5 sat. $MgSO_4$. Dissolve in N/50 H_2SO_4. Bring to 0.5 sat. with $MgSO_4$. Add 0.4 M borate to give a pH of 9.0. Let stand 1 day at 5° C. and filter off the crystals of trypsin. Yield, about 6 g. from 7 liters of acid extract of the pancreas.

4. *Preparation of Vegetable Lipase: Procedure of Willstätter and Waldschmidt-Leitz.* Rub in a mortar to a paste 20 g. hulled castor beans.[19] Then with continued rubbing add 140 ml. of water in portions of 5 to 10 ml. Centrifuge for 15 minutes at 3,000 revolutions per minute. Three layers are formed. Pour off the upper creamy layer and retain. Pour off the clear water layer and discard. Rub up the residue with 140 ml. of water as before and centrifuge. Pour off the creamy top layer and combine with the first portion. Use this suspension for lipase experiments.

5. *Preparation of Sucrase from Yeast.* Introduce 100 g. of compressed yeast into a 400-ml. beaker. Warm the yeast to 30° C. by placing the beaker for some time in a water bath at a slightly higher temperature. Add 10 ml. of toluol. Stir thoroughly with a heavy glass rod. The yeast should become liquefied in 30 to 45 minutes. Add 200 ml. of water. Mix thoroughly and centrifuge. To the residue in the centrifuge tubes, add a small amount of water and mix well, then add more water at 30° C. to make a total of 200 ml. Stir and centrifuge. To the yeast residue after pouring off the water, add 100 ml. of water saturated with toluol and 10 ml. of toluol, and incubate at 30° C. over night. Dilute with 4 volumes of water. With vigorous stirring, carefully add acetic acid (not over 1 N) until a test with methyl red indicates a pH of about 3.5 to 4.0. Centrifuge. To the supernatant fluid add some infusorial earth and filter. Neutralize with ammonia to complete change of color with bromocresol purple. Keep in a refrigerator.

6. *Preparation of Crystalline Catalase.*[20] Put fresh beef liver through a meat grinder four times. Extract the catalase at room temperature by the occasional shaking of each kilogram of ground liver for 10 min. with 1 l. of distilled water. To each liter of material add 480 ml. of chilled solution of chloroform, 1 part; and ethyl alcohol, 2 parts. Shake violently for about 30 sec. and at once filter in the ice chest through Whatman No. 12 filter papers. The next day the filtrates will contain a sediment of catalase crystals. This sediment is centrifuged down in a refrigerated centrifuge and the supernatant liquid is discarded. The crystals are dissolved at room temperature in distilled water (30–40 ml. for each kilogram of liver used). The solution is

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[19] The greatest care must be taken to avoid poisoning by the ricin which is present in the meats.

[20] Mosimann: *Arch. Biochem. Biophys.*, 33, 488 (1951).

centrifuged to remove a white amorphous impurity, decanted, and chilled in the ice chest for 12 to 24 hr. Catalase will precipitate as needles.

QUANTITATIVE ESTIMATION OF ENZYME ACTION

The amount of enzyme present in any mixture is expressed in terms of its activity as compared with an arbitrary standard. Either the time required for a given amount of enzyme preparation to bring about a definite degree of conversion of the substrate or the amount of preparation needed to bring about a definite degree of conversion in a specified time may be made the basis of comparison. Inasmuch as enzyme action is greatly influenced by the pH of the solution, by the presence of inorganic salts and activators, and by temperature, it is important that the conditions in the digestion mixtures should be made as nearly identical as possible and that the enzyme if in inactive form should be properly activated.

Chemical reactions follow the law of mass action—that is, their speed is proportional to the product of the concentrations of the reacting substances. In most enzyme reactions the speed of decomposition of the substrate tends at first to be directly proportional to the amount of enzyme present, particularly if the substrate is present in large excess. Later the reaction may slow up due to decrease in the amount of substrate or combination of the enzyme with substrate or products of the reaction. In quantitative studies of enzyme action it is best to have a large excess of substrate and to keep the time of the experiments within the period when decomposition is proportional to time. To establish these limits an experiment must be conducted and a curve plotted showing the extent of decomposition at different time intervals. In certain cases, direct proportionality does not exist even in the early stages. In such cases a curve prepared as above will suggest the rule to be applied in the calculation of results.

DEMONSTRATION OF THE REVERSIBLE ACTION OF AN ENZYME

The enzyme phosphorylase is found in plant and animal tissues. This enzyme catalyzes the following reaction:

$$\text{glycogen (or starch)} + \text{inorganic phosphate} \rightleftarrows \text{glucose-1-phosphate}$$

The reaction is reversible and either glycogen or starch can be used to form glucose-1-phosphate (Cori ester). Whether glycogen or starch is formed from glucose-1-phosphate depends upon the source of the enzyme. Muscle phosphorylase (*in vitro*) and the phosphorylase from higher plants form a polysaccharide which is probably identical with the amylose fraction of starch. Yeast phosphorylase and the phosphorylase from liver, brain, and heart form glycogen from glucose-1-phosphate. In this case a second enzyme, the Q enzyme, is present in addition to phosphorylase. This Q enzyme forms 1–6 linkages.

The position of the equilibrium is influenced by pH. At pH 7 an equilibrium is obtained when the total phosphate is 23 per cent as Cori ester and 77 per cent as inorganic phosphate.

1. *Formation of Glucose-1-phosphate* (*Hanes method as modified by Sumner and Somers*). Boil 8 g. of soluble starch with about 100 ml. of water. Cool and add 12 g. of Na_2HPO_4 and 5 g. of KH_2PO_4 dissolved in about 300 ml. of water. Add 100 ml. of potato-cyanide extract. Dilute to 1 liter, add toluene, and mix. Keep at 20° to 25° C. for about 24 hours.

Inactivate the phosphorylase by adding 0.1 N iodine until the solution gives a permanent reddish-brown color. Remove the iodine by adding 0.1 N thiosulfate until all brown color has disappeared. Add 10 to 20 ml. of 2 per cent pancreatin and allow to digest until no more dextrin remains, as shown by the iodine test. (This will take three or four hours.) Add 40 g. of barium acetate and about 8 ml. of 28 per cent ammonia, or enough to make the suspension definitely alkaline to phenol red. Mix well, centrifuge, and pour the supernatant through cotton. To each volume of supernatant add 2 volumes of 95 per cent alcohol and centrifuge down the barium salt of glucose-1-phosphate. Discard the supernatant. Stir the precipitate with 30 to 60 ml. of water and enough 2 N sulfuric acid to give a pink color with thymol blue paper. Now add saturated potassium hydroxide cautiously until the suspension just fails to give a blue or brown color with Congo red paper. Add 6 g. of trichloroacetic acid and mix. To every volume of the suspension add 2 volumes of 95 per cent alcohol and stir. Centrifuge down the suspended matter. Decant the clear supernatant solution. Add saturated potassium hydroxide to it until it is decidedly alkaline to phenol red. The di-potassium salt of glucose-1-phosphate usually separates as an oil. Chill overnight at 0° to 5° C. Next day filter off the crystals, wash several times with 95 per cent alcohol and then with acetone, and dry at 50° C. The yield will be about 3.5 g., and the product will be about 85 per cent pure.

The potato-cyanide is prepared by disintegrating about 325 g. of recently sliced potato in 100 ml. of 0.01 N neutralized potassium cyanide in a blender. The disintegrated mass is squeezed in cheesecloth and the juice is centrifuged to eliminate the starch.

The hexose-1-phosphate, or Cori ester, is $C_6H_{11}O_9PK_2 \cdot 2H_2O$. Does a trace of it reduce Benedict's solution? Heat a trace of it in boiling water for 5 minutes with 1 ml. of N sulfuric acid. Neutralize with 0.1 N NaOH. Now test for glucose. Test also for inorganic phosphate.

2. *Formation of Starch from Glucose-1-Phosphate.* Prepare 5 to 10 ml. of 0.1 per cent glucose-1-phosphate in water. Add a drop of phenol red and enough 0.1 N hydrochloric acid to bring the alkaline solution to approximate neutrality. Place 1 ml. of the solution in a test tube and add about 1 ml. of potato-cyanide extract. Add a drop of 0.01 per cent boiled starch solution and mix. This primes the reaction. From time to time remove a drop or two of the digest and test on a porcelain spot plate by adding a few drops of 0.01 N iodine solution. Is starch formed? What is the chemical reaction?

CELL RESPIRATION

The ultimate objective in the study of those enzymes which are found within cells is to apply this knowledge to an understanding of the metabolic processes upon which the cell depends for its maintenance and function. The contributions of enzyme chemistry to this subject have been numerous and important, but it is clear that the isolation of an enzyme or enzyme activity from cells does not necessarily give information as to the significance of the enzyme in the intricate processes of metabolism within the cell. An alternate method of approach, therefore, is to study the metabolic behavior of the isolated intact cell or tissue, under as nearly physiological conditions as possible, and to integrate knowledge gained in this way with that acquired by the study of individual enzyme systems.

It was Warburg who first showed that animal tissues and organs (liver, kidney, brain, etc.) could be prepared in the form of thin sections or minces which would continue to carry on metabolic processes (respiration, substrate utilization) for many hours after removal from the animal body if placed under suitable conditions, and that such metabolism could be followed quantitatively. While it is obvious that cells under these circumstances are no longer under the control of nervous or hormonal mechanisms, metabolic data obtained by this method of approach do not disagree with results obtained on the intact animal where the two methods can be compared, and have furnished valuable information concerning the localization of specific metabolic processes in individual organs of the animal body, as well as the effect of different substrates, coenzymes, activators, inhibitors, etc., on cell metabolism.

In the study of isolated cells and tissues, chief attention has been directed to the respiration, and specifically to the oxygen consumption. In fact, some have defined the respiration of cells in terms of oxygen consumption alone; but carbon dioxide production is an equally important phase of respiration, and any complete characterization of cell respiration must include both oxygen consumption and carbon dioxide production. In addition to respiratory data, much valuable information has been obtained by the use of the Warburg tissue-slice technique concerning other metabolic characteristics of cells, such as substrate utilization, fermentative (glycolytic) ability, glucose and glycogen synthesis, urea formation, conjugation of foreign organic compounds, etc. The discussion in these pages will be confined to respiration and glycolysis, two characteristics of cell metabolism which are readily measured manometrically; but it cannot be too strongly emphasized that knowledge of this type is essentially incomplete until it is supplemented with precise information concerning substrate utilization and end-product formation; this fact should be more generally recognized.

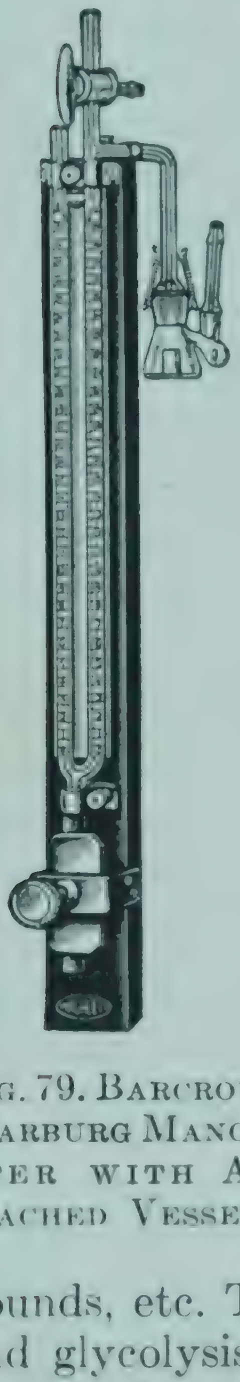

FIG. 79. BARCROFT-WARBURG MANOMETER WITH ATTACHED VESSEL.

Measurement of Oxygen Consumption. The basis of the Warburg method for the measurement of the oxygen consumption of living cells is the apparatus shown in Fig. 79. This is sometimes referred to as the Warburg apparatus, but more properly is called the Barcroft-Warburg apparatus, since it was adapted by Warburg from that developed by Barcroft for the study of blood gases. It consists of a suitable vessel, containing the material to be studied in the proper fluid medium, and attached in a closed system to a manometer for measuring changes in gas pressure.[21] Various types of vessels in common use are shown in Fig. 80; many other types have been described for special purposes. In general, an ordinary Barcroft-Warburg vessel has a capacity of 15 to 20 ml., with a center well and one or more side bulbs; larger and smaller vessels have also been used. The type of bored side-bulb plug illustrated is much to be preferred over the more common solid plug.

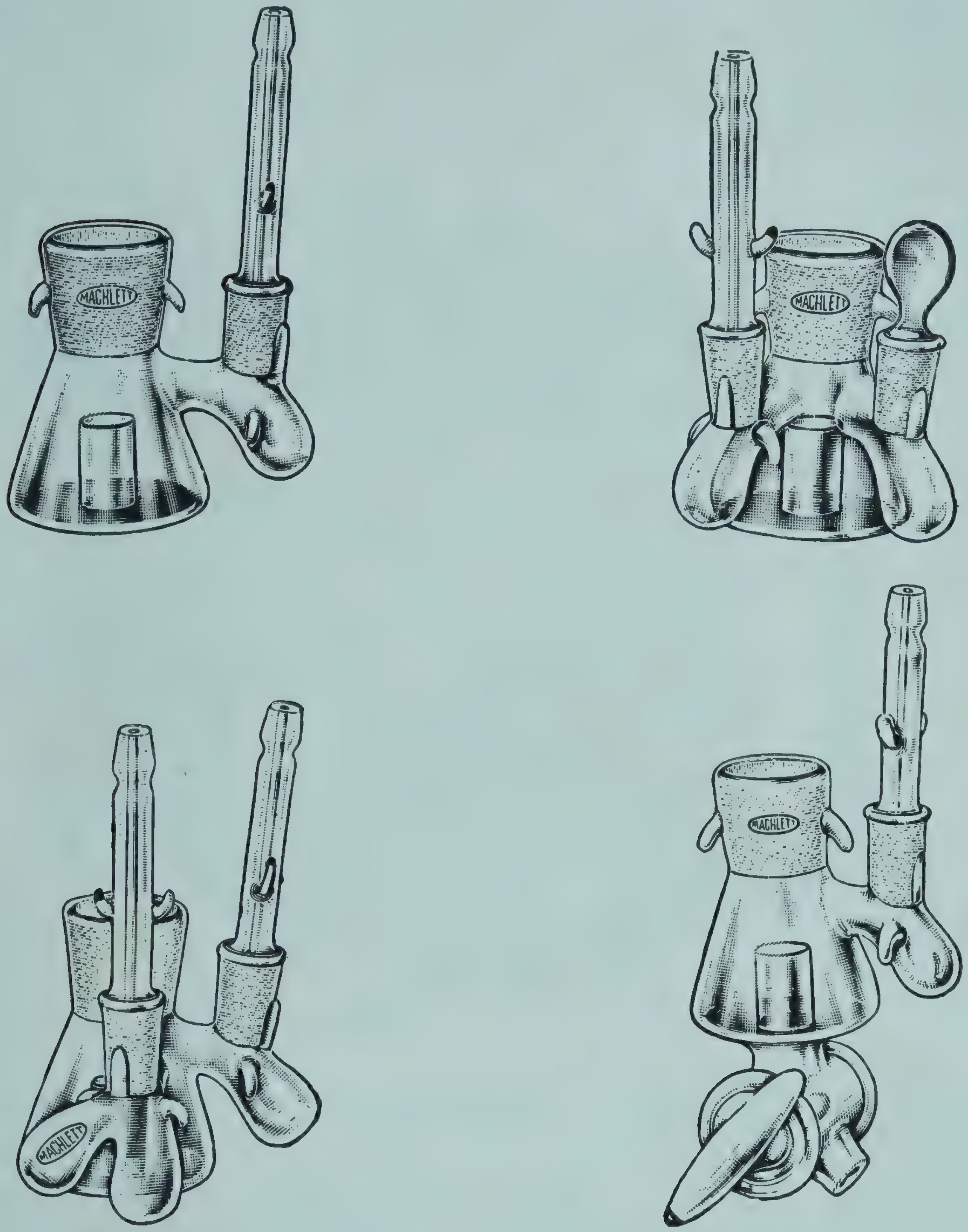

FIG. 80. TYPES OF VESSELS USED FOR MANOMETRIC STUDIES ON CELL RESPIRATION.

To provide for accurate temperature control, manometers and vessels are so constructed that the manometer may be mounted on the side of a constant-temperature water bath (thermostat), with the vessel completely immersed in the water. To ensure equilibration between fluid medium and gas phases in a vessel during an experiment, the manometer mounting is attached to a shaking device which shakes vessel and manometer horizontally at speeds which ordinarily amount to 110–115 oscillations per minute, with an excursion of 3–4 cm. A complete assembly of this type is illustrated in Fig. 81.

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[21] Complete equipment of the type described here (manometers, glassware, and thermostat) may be obtained from E. Machlett and Son, New York City; Arthur H. Thomas Co., Philadelphia; or American Instrument Co., Silver Springs, Md.

The detailed application of the Warburg procedure for the measurement of oxygen consumption is given in the experiments which follow this discussion. The principle is as follows: The tissue, usually in the form of thin slices, is incubated at body temperature in a suitable buffered medium, in a vessel with attached manometer, the vessel containing oxygen rather than air. The center well of the vessel contains a little strong alkali solution which absorbs any CO_2 produced, so that any pressure changes are due to oxygen consumption alone. The side-bulbs provide for the addition of substrate, activators, inhibitors, etc., during an experiment if desired. The gas phase in vessel and manometer capillaries is kept at constant volume; oxygen consumption is measured therefore by a fall in pressure, which is read on the manometer. The pressure readings when multiplied by a constant ("vessel constant") give the oxygen consumption, usually expressed in microliters (cubic millimeters). Readings are made at suitable intervals; they may be plotted graphically against time, or may be averaged to give the oxygen consumption over a given time interval. The standard method for expressing oxygen consumption is in terms of the number of microliters consumed in one hour by 1 mg. (dry weight) of tissue; this is symbolized by Q_{O_2}. By convention, the consumption of a gas is given a negative value. Thus a Q_{O_2} of −6.0 represents the consumption of 6 microliters of oxygen per hour by 1 mg. dry weight of tissue.

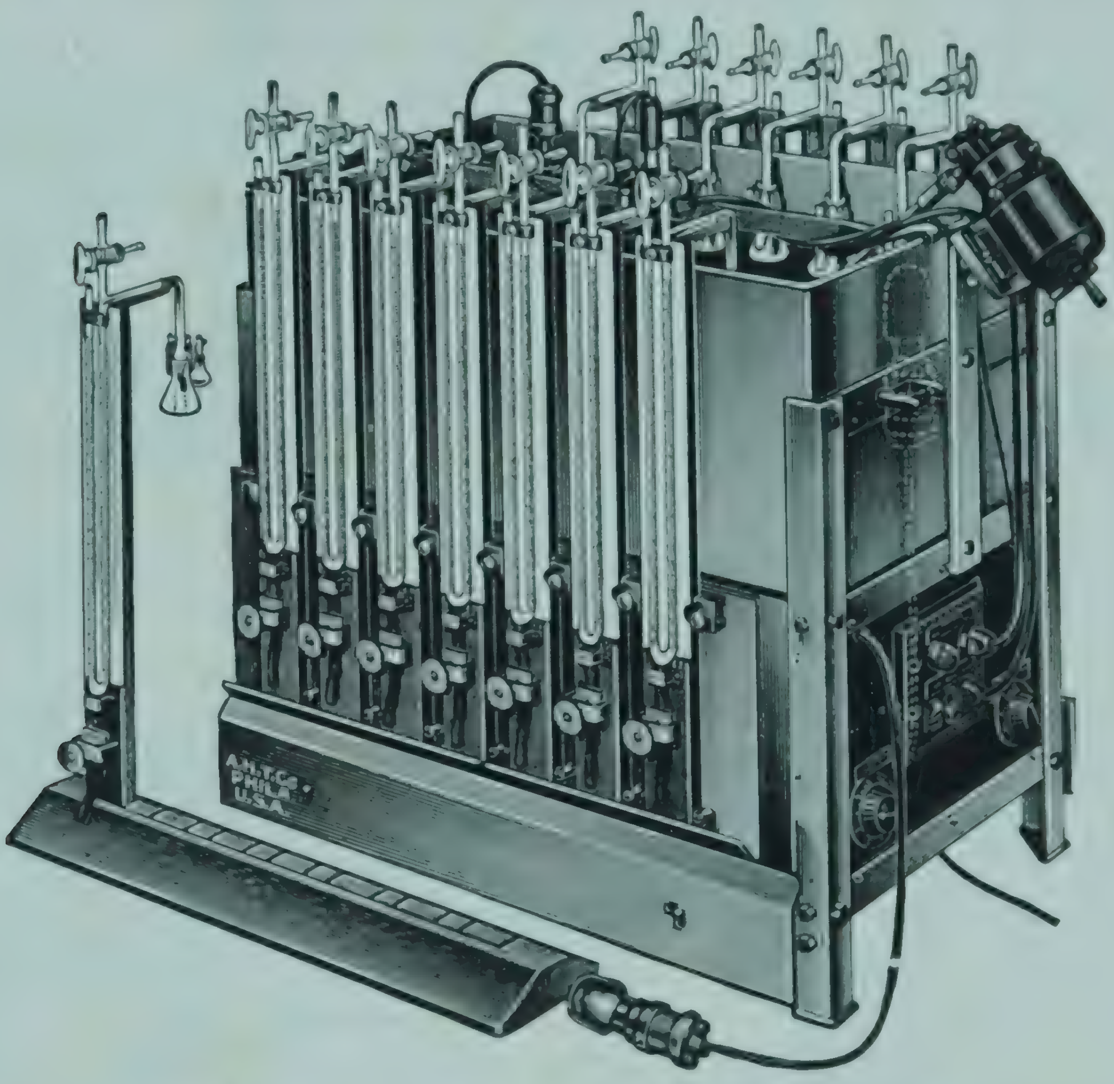

FIG. 81. ASSEMBLY OF CONSTANT-TEMPERATURE BATH AND MANOMETERS AS USED FOR STUDIES ON CELL RESPIRATION.

Representative Q_{O_2} values for various animal tissues are given in the table on p. 340. These values must be considered approximations only, since it is known that the oxygen consumption of many tissues is affected by: the presence or absence of various substrates, the previous nutritional history of the animal, the inorganic ion composition of the medium and the type of buffer used, the length of elapsed time between the death of the animal and the start of an experiment, and even the duration of the experiment itself, since oxygen consumption may not be linear with respect to time. Despite these limitations, many valuable data have been obtained by this procedure, particularly where comparative and not absolute values are desired, as in studies on the relative effects of various substrates, coenzymes, and inhibitors, comparison between normal and abnormal tissues, etc.

A more serious limitation in the Warburg method, and one which is receiving increasing recognition, is that the tension of CO_2 must be maintained at or near zero during the experiment; otherwise manometer readings will not reflect changes in oxygen content alone. This requirement means that metabolism is proceeding in the virtual absence of CO_2, and it is known that certain metabolic processes (e.g., the formation of urea by liver cells) require the presence of CO_2; even the oxygen consumption of some tissues is significantly affected by the presence or absence of a physiological tension of CO_2. Furthermore, measurements by the Warburg method are not possible in such media as normal blood serum or other bicarbonate-containing media, since such media require the maintenance of a finite tension of CO_2 to establish the pH.[22] For the measurement of oxygen consumption and other respiratory characteristics of tissues in the presence of physiological tensions of CO_2, the differential manometer described in the next section in connection with the measurement of CO_2 production must be used.

Measurement of Carbon Dioxide Production. Measurement of carbon dioxide production by respiring cells or tissues is technically more difficult than is measurement of oxygen consumption. There is no satisfactory direct method for the continuous measurement of the carbon dioxide produced by cell respiration, as there is for the measurement of oxygen consumption. Hence relatively little work has been done on this phase of respiration. This is unfortunate, for, as we now know, carbon dioxide is produced not by the direct oxidation of carbon compounds by oxygen but rather by decarboxylation of organic acids, and in a manner essentially independent of oxygen consumption but presumably of equal importance to the cell. Carbon dioxide production usually is expressed by giving the value of the respiratory quotient (R.Q.), which is the ratio of the volume of carbon dioxide evolved to the volume of oxygen consumed in the same time; hence if the Q_{O_2}, and R.Q. are given, the carbon dioxide production is defined.

One method for the determination of R.Q. in phosphate buffer using the ordinary Barcroft-Warburg manometers is to set up duplicate tissue preparations but to omit the alkali from the center well of one vessel. Pressure changes in the vessel containing alkali are due to oxygen consumption alone; pressure changes in the second vessel, without alkali, represent the net (and opposing) effects of oxygen consumption and carbon dioxide production. At the end of the experiment, by subtracting the pressure change due to oxygen consumption alone, as established on the first manometer, from the reading of the second manometer, the pressure change due to carbon dioxide production may be obtained. Conditions in the two vessels, including vessel constants, must be as nearly alike as possible. This, of course, cannot be true with respect to the CO_2 tension, which is zero in the first vessel and continuously increasing in the second vessel. Results will therefore be in error if the CO_2 tension influences metabolic processes within the cell, and this is known to be the case with a number of different types of cells. This method is therefore not widely used.

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[22] For measuring oxygen consumption by the Warburg method in "neutralized" serum—i.e., serum which has been freed of bicarbonate by treatment with acid and evacuation—see Macleod and Rhoads: *Proc. Soc. Exptl. Biol. Med.*, **41**, 268 (1939); Warren: *Am. J. Physiol.*, **128**, 455 (1940).

It is also possible to so arrange matters that only one vessel containing alkali is used, and the CO_2 absorbed by the alkali is measured by liberating with acid at the end of the experiment. This method is technically quite difficult, is not too accurate, and likewise suffers from the disadvantage that measurements are made in the presence of a zero CO_2 tension.

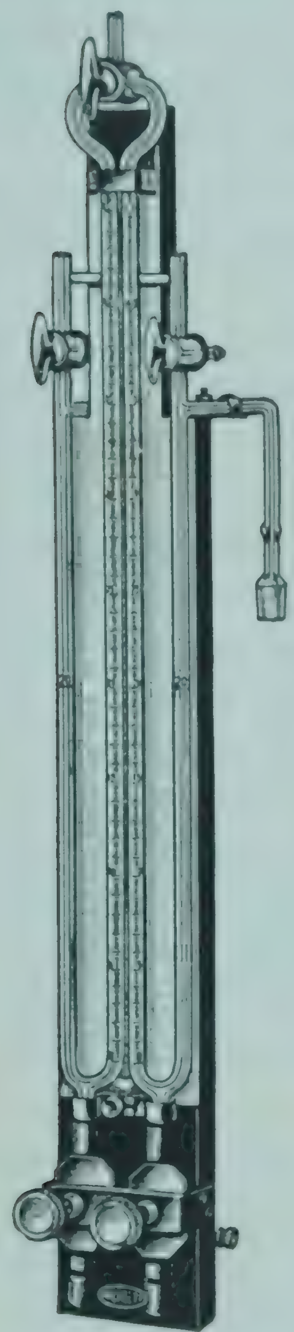
FIG. 82. CONSTANT-VOLUME DIFFERENTIAL MANOMETER.

To permit measurement of carbon dioxide production (and oxygen consumption as well) in the presence of a physiological tension of carbon dioxide, and therefore in media such as blood serum or Ringer-bicarbonate solution which are more nearly physiological than phosphate or similar buffers, various types of so-called differential manometers have been developed. One such type[23] is illustrated in Fig. 82. It consists of two independent vessels and manometers, with the manometers so arranged that pressure changes in the two vessels may be opposed to one another on the manometer, to permit precise measurement of the pressure differences between the vessels rather than the total pressure in each. In use, the two vessels are charged with identical amounts of medium and tissue, the medium being either blood serum or Ringer-bicarbonate, and the gas phase being oxygen containing a physiological concentration (usually 5 per cent) of carbon dioxide. At the beginning of the experimental period, the tissue in one vessel is killed by tipping in acid from a side bulb, and the tissue in the other vessel is then allowed to respire for the desired length of time, after which it too is killed by the addition of acid. Although the total pressure in both vessels is now quite high, due to the liberation of CO_2 from the decomposed bicarbonate in the medium, by opposing one vessel pressure against the other on the manometer only the difference in pressure caused by the respiration of the tissue in the second vessel during the experimental period will register on the manometer. This pressure difference is due to both oxygen consumption and carbon dioxide production. After it is noted, alkali is admitted into both vessels through a bottom stopcock (see Fig. 80 for the type of vessel used) and all the carbon dioxide is absorbed. Again the differential reading is noted; this is now due solely to oxygen consumption by the respiring tissue. The carbon dioxide production is obtained by subtracting this reading from the first differential reading. Thus the oxygen consumption and R.Q. are accurately measurable under a continuous physiological tension of carbon dioxide. The only disadvantage of this method over the simple Warburg method as far as oxygen consumption measurements are concerned is that the Warburg method gives continuous readings, whereas the differential method gives but one reading, representing the entire experimental period. Conditions, therefore, must be selected so that respiration is linear with respect to time, or nearly so.

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[23] Summerson: *J. Biol. Chem.*, **131**, 579 (1939).

The use of the differential manometer is technically somewhat more difficult than is that of the simple Barcroft-Warburg manometer, but it is the only accurate basis for the measurement of cell respiration under conditions which closely simulate those prevailing normally, and the increased significance of the results obtained more than justifies the technical difficulties.

A further advantage of the differential manometer is that it permits the measurement of aerobic acid production (*aerobic glycolysis*, see below) by respiring tissues. If the tissue produces acid—e.g., lactic acid—aerobically in a medium containing bicarbonate, neutralization of the acid results in the decomposition of an equivalent amount of bicarbonate to produce gaseous CO_2. This CO_2 is indistinguishable manometrically from the CO_2 of respiration unless the differential manometer is used, where it is readily done by a simple method which need not be described here (for details see Summerson (*loc. cit.*) and also books by Dixon and by Umbreit, et al., cited in the Bibliography). Since the production of acid aerobically is a characteristic which differentiates tumor tissues from many (but not all) normal tissues (see table on p. 340), the differential manometer is useful in metabolic studies on such tissues.

Anaerobic and Aerobic Glycolysis. Practically all tissues show the ability to produce lactic acid from either glucose or glycogen under anaerobic conditions. Such production of lactic acid is known as glycolysis, although strictly speaking this term implies merely a disappearance of carbohydrate rather than the specific production of lactic or other acids. With certain tissues—e.g., liver, kidney—the anaerobic formation of lactic acid is at the expense of tissue glycogen and is independent of the presence of glucose; it has been proposed that the term *glycolysis* be restricted to such origin of lactic acid from glycogen, and that the term *glucolysis* be used where glucose is the source of the lactic acid.

Anaerobic glycolysis by tissues is readily measured with the simple Barcroft-Warburg manometers by suspending the tissue in a bicarbonate-containing medium in equilibrium with the proper tension of CO_2 in the gas phase to maintain a suitable pH, but with nitrogen rather than oxygen present. Since in the absence of oxygen there is no respiration, pressure changes on the manometer will ordinarily be due solely to decomposition of bicarbonate by the acid produced anaerobically. Anaerobic glycolysis usually is represented by the symbol $Q_G^{N_2}$, and is expressed in terms of microliters of CO_2 produced by the action of acid on the bicarbonate present, per milligram dry tissue per hour. The anaerobic glycolytic power of various animal tissues is given in the table below. No distinction is made in this table between glucolysis and glycolysis, and the values for certain tissues such as liver and kidney are subject to considerable variation because the rate of glycolysis usually falls off continuously throughout the average experimental period. It will be noted that various tissues differ considerably in their $Q_G^{N_2}$ values, and that tumor tissues in general (but not exclusively) are quite high in this respect.

RESPIRATION AND GLYCOLYSIS OF SELECTED ANIMAL TISSUES*

Tissue	Q_{O_2}	*R.Q.*	$Q_G^{O_2}$	$Q_G^{N_2}$
Muscle (dog)	2	0.95	0	4
Pancreas (dog)	3	..	0	4
Bone marrow (rabbit)	5	0.90	2	13
Rous sarcoma (chicken)	5	0.93	20	30
Liver (fetal, rat)	7	1.00	0.5	8
Testis (rat)	8	0.90	4	8
Jensen sarcoma (rat)	9	0.78	17	34
Liver, (adult, rat)	10	0.5–1.0	0.5	3
Embryo (chicken)	11	1.00	2	18
Intestinal mucosa (rat)	12	0.85	2	4
Spleen (rat)	12	0.89	2	8
Brain (rat)	13	1.00	2	19
Thyroid gland (rat)	13	..	0	2
Retina (rat)	19	1.00	22	88
Kidney (rat)	21	0.83	0	3
Chorion (rat)	26	1.00	7	32

* Many values are averages from the literature. For significance of symbols used, and further discussion, see text.

The production of acid aerobically is also measurable in terms of the decomposition of bicarbonate, is symbolized by $Q_G^{O_2}$, and expressed in the same units as for anaerobic glycolysis. Aerobic glycolysis is more difficult to measure than anaerobic glycolysis, since there must be a way to differentiate the CO_2 of acid production from the respiratory CO_2. The only successful way to do this is with the differential manometer, as discussed previously. In contrast to acid production anaerobically, which in the majority of cases is quantitatively due to lactic acid, only a fraction of the acid produced aerobically is usually accountable for as lactic acid. Little specific information is available concerning the nature of other acids produced under these circumstances. As can be seen from the data above, most normal adult tissues have a relatively low aerobic glycolysis; certain specialized normal adult tissues, and all tumor tissues, are characterized by a significant aerobic glycolysis.

It is felt by some that the inhibition of anaerobic glycolysis ("fermentation") by aerobic metabolism, which is called the *Pasteur effect,* may be associated with the presence of a specific enzyme (*Pasteur enzyme*) whose function is to orient cell metabolism into either fermentative or oxidative pathways. Evidence for the existence of such an enzyme is limited at the present time.

EXPERIMENTS ON CELL RESPIRATION

1. Calibration of Vessel and Manometer: Principle. For the calculation of changes in gas content in a vessel from the manometer readings, under a particular set of experimental conditions, it is necessary to know the entire volume of gas space in the empty vessel, manometer side-arm, and manometer capillary down to the level of manometer fluid as it will be placed in actual use. The best way to do this is by filling the entire space with mercury, then collecting and weighing the mercury; from the known density of the mercury at the temperature of use, the volume occupied by the mercury is readily obtained.

Procedure. **(a) Place the manometer (clean, dry, and empty of manometer fluid) in an upright position. Fill the clean dry vessel with mercury, being sure that the vessel side tap is firmly in place (it may be lightly greased and held secure by springs or wound rubber bands). The mercury must fill the entire vessel including the center well and side bulb; any trapped air bubbles may be dislodged with a piece of fine wire. The mercury should come to within about one-half inch of the top rim of the vessel. Holding the vessel in the hand, set it slowly in place on the manometer side arm until it is firmly seated. Excess mercury will run out between vessel and manometer ground-glass surfaces during this process, should be brushed off if adhering to the glass, and may be caught in a large tray set beneath the entire assembly for this purpose. A thread of mercury will likewise be forced up into the capillary of the manometer side arm for a variable distance. When the vessel is firmly seated on the manometer, make a mark on the manometer side arm to indicate the limit of the mercury thread. The best position for this is in the straight portion of the side-arm capillary a few centimeters above the vessel; other vessels may then be calibrated on this same manometer in the future, without the necessity of removing any manometer fluid present, by adjustment of the mercury thread to the same mark; this adjustment is particularly easy if the vessel side-bulb tap is bored as shown in Fig. 80, since by turning the tap to open it slightly, by slight pressure or suction the mercury level in the manometer side-arm capillary may be controlled at will.**

When the mark has been made, slowly withdraw the vessel from the manometer, allowing the capillary thread of mercury to follow and be caught in the mercury in the vessel as it is removed from the manometer. Transfer the mercury from the vessel to a tared beaker, and weigh the mercury to the nearest 0.1 g. Note the temperature of the mercury. From the volume occupied by 1 g. of mercury at the indicated temperature,[24] calculate the

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[24] The volume occupied by 1 g. of mercury at various temperatures is as follows:

Temp. °C.	*Volume ml.*	*Temp. °C.*	*Volume ml.*
16	0.07377	24	0.07388
18	0.07379	26	0.07390
20	0.07382	28	0.07393
22	0.07385	30	0.07396

volume occupied by the mercury at this stage of the calibration. Call this volume V_V.

(b) Invert the manometer, turn it so that the front faces to the right and the side arm projects to the left, and fasten it securely in an upright position with a large adjustable buret clamp attached to a ring stand. Now loosen the attachment of the buret clamp to the ring stand and tilt the manometer clockwise until the calibration mark on the side arm and the 150-mm. graduation mark on the attached manometer limb are in line with each other and parallel to the table top. Clamp the manometer firmly in this position. Attach to the side orifice of the manometer stopcock a piece of rubber tubing carrying a screwcock and connected to a leveling bulb containing mercury. The manometer stopcock should be lightly greased and may be held secure by rubber bands. Turn the manometer stopcock so that mercury may enter from the rubber tubing and rise into the manometer capillaries. By carefully releasing the screwcock on the rubber tubing, allow mercury to enter the manometer capillaries and rise in the side arm and main limb until the mercury level reaches both the calibration mark on the side arm and the 150-mm. graduation on the main limb. Slight further tilting of the manometer may be necessary at this point. If there are any trapped air bubbles, lower the leveling bulb to retract the mercury and repeat the adjustment.

When both capillaries of the manometer are filled with mercury to the desired points, turn the manometer stopcock very slightly to cut off the flow of mercury, place a tared beaker under the straight capillary orifice of the manometer stopcock, and then turn the stopcock so that the mercury drains from the manometer capillaries into the beaker. Any droplets of mercury remaining behind may be forced out and into the beaker by blowing down the capillaries. Weigh the mercury, determine its temperature, and calculate the volume of the side-arm and main limb capillaries. Call this volume V_M.

CALCULATION. The total volume of empty vessel and manometer gas space, V_T, is equal to $V_V + V_M$. From the value of V_T, the vessel constant k under a particular set of experimental conditions is calculated as follows:

$$k = \frac{V_T}{P_o} \cdot \frac{273}{T} - \frac{V_F}{P_o}\left(\frac{273}{T} - \alpha\right)$$

where V_T is the total volume of vessel and manometer, as described above, and V_F is the volume of fluid medium in the vessel during an experiment; both of these volumes are expressed in microliters (cubic millimeters) rather than in milliliters (1 ml. = 1000 microliters). P_o is the equivalent of 1 atmosphere pressure in mm. of manometer fluid (760 for mercury, 10,000 for Brodie fluid; see below), T is the temperature in degrees Absolute at which measurements are made, and α is the absorption coefficient (solubility) of the gas undergoing absorption or evolution.[25] For the derivation of this equation, see the book by Dixon; the form of the equation is that described by Macleod and Summerson.[26]

It can be seen that the constant for a particular vessel and manometer depends upon the size of the vessel, the volume of medium used, the temperature, and the nature of the gas concerned. The following example illustrates the calculation of the vessel constant for oxygen, k_{O_2}, for a particular vessel and manometer where V_T = 15.75 ml. and 2.0 ml. of medium are employed, at 38° C.

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[25] At 38° C., α for O_2 is 0.024; for N_2, 0.012; and for CO_2 in water 0.55; in Ringer solution, 0.537, and in Ringer solution containing 0.3 N HCl, 0.517. For values of other gases and at other temperatures, see handbooks giving physical constants of gases.

[26] Macleod and Summerson: *Science*, **91**, 201 (1940).

$$V_T = 15.75 \text{ ml.} = 15{,}750\ \mu\text{l.}$$
$$V_F = 2.00 \text{ ml.} = 2{,}000\ \mu\text{l.}$$
$$T = 38° \text{ C.} = 311° \text{ A.}$$
$$P_o = 10{,}000$$
$$\alpha_{O_2} = 0.024 \text{ at } 38° \text{ C.}$$

therefore

$$\begin{aligned} k_{O_2} &= \frac{15{,}750}{10{,}000} \times \frac{273}{311} - \frac{2{,}000}{10{,}000}\left(\frac{273}{311} - 0.024\right) \\ &= 1.38 - 0.17 \\ &= 1.21 \end{aligned}$$

The vessel constant will be different with other amounts of medium, at other temperatures, and for other gases. For routine work on oxygen consumption at 38° C., the only variable is likely to be the volume of medium employed. Vessel constants should therefore be calculated for the various volumes of medium apt to be used, or the simple graphical method described by Macleod and Summerson (*loc. cit.*) for this purpose may be employed.

The calibration is described for a manometer fluid setting at the 150-mm. mark on the graduated limb. This is the most satisfactory position, but calibration obviously can be made to some other setting. If a thread of mercury is placed in the graduated capillary, the length in mm. noted, and the mercury then weighed and its volume computed, the volume of the graduated capillary per mm. may be obtained, and from this it is possible to calibrate the manometer at any setting on the scale provided the calibration at one setting is also known. This is of value under certain circumstances.

MANOMETER FLUID. The most commonly used manometer fluid is the aqueous salt solution described by Brodie[27] with a specific gravity of 1.033, so that 10,000 mm. are equal to 1 atmosphere (760 mm. of mercury); this makes for obvious convenience in calculation, with a much greater sensitivity than mercury. For special purposes, however, any other fluid whose P_o value is known (water, mercury, paraffin oil, etc.) may be used.

The manometer fluid usually is contained in a stoppered rubber tube attached to the manometer which acts as a reservoir, controlled by the pressure of a screwcock. The fluid may gradually leak out or be otherwise lost; it may be replenished at any time, even during an experiment, by inserting a hypodermic needle attached to a syringe containing extra fluid through the rubber tube wall, at a slight angle to form a "Bunsen valve" after withdrawal of the needle. It may also be added through the open top of the manometer, with precautions to avoid trapping air bubbles. Should such bubbles be present, they may be forced to the top of the fluid column and dissipated by alternate pinching and release of the rubber tube reservoir with the fingers, to force the manometer fluid up and down.

2. Measurement of Oxygen Consumption of Rat Liver Slices by Warburg: Method. (a) PREPARATION OF TISSUE. **Kill a young adult rat by a blow on the**

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[27] Brodie fluid is made as follows: Dissolve 23 g. of sodium chloride and 5 g. of bile salts (sodium tauroglycocholate, or sodium glycocholate, Merck) in 500 ml. of water. A few drops of an alcoholic thymol solution may be added as a preservative, and it is convenient to color the fluid by adding a few hundred mg. of a suitable dye (Crystal violet, Gentian violet, or Evans blue). The specific gravity of the final solution should be 1.033; i.e., 10 ml. should weigh 1.033 times as much as 10 ml. of water at the same temperature.

head, followed by severing the neck vessels and spinal cord at the neck with scissors. Open the abdominal cavity and remove the liver as quickly as possible. Rinse the liver in a beaker containing Ringer's solution,[28] then cut off the largest lobe of the liver and place flat on a pad of filter paper to drain momentarily. With a sharp thin razor blade moistened with Ringer's solution, cut off a small portion of the liver in such a way that the cut surface makes an angle of about 45° with the table top, and discard this portion. Continue cutting freehand along the plane of the first cut surface to obtain a number of thin slices of liver, making each slice as uniformly thin as possible (the leading edge of the razor blade should be visible through the slice as it is being cut). With practice, uniform slices about 0.5 mm. thick are readily obtained. As each slice is obtained, transfer it to a flat dish containing Ringer's solution, and keep the blade of the razor and the cut surface of the liver moistened with Ringer's solution. Continue cutting until sufficient slices for the experiment are obtained; usually two to four slices are required per vessel.

(b) PREPARATION OF VESSELS. Measure duplicate (or triplicate) 2-ml. portions of medium[29] into clean dry Barcroft-Warburg vessels, avoiding the center well. In another vessel place a few ml. of water; this will be the thermobarometer control. With a forceps or platinum wire, transfer a liver slice from the dish to a pad of filter paper and drain momentarily, then quickly transfer to a tared watch glass on a balance. Add more slices treated in the same way until the desired weight of liver tissue is obtained; 100 to 200 mg. wet weight is usually satisfactory. Immediately transfer the weighed clump of slices to one of the vessels, immersing them in the medium. The vessel may be shaken briefly to separate the slices. In like manner, charge the remaining vessels containing medium with weighed amounts of tissue. The weights of tissue in each vessel need not be identical, but they should be known to the nearest milligram.

When all the vessels are ready, obtain one more known weight of tissue by exactly the same procedure, but transfer this to a small tared watch glass or weighing dish. This is to be dried in an oven overnight at 100° C., and weighed again, to determine the dry weight of the tissue.

When the tissue is in place in the vessels, complete the preparation of the vessels by placing in the center well of each (except the thermobarometer vessel) 0.2 ml. of 10 per cent KOH solution, using a pipet with a fine tip. No alkali must get into the medium surrounding the center well. It is advantageous, but not absolutely necessary, at this point to insert a small roll of starch-free filter paper (Whatman No. 40 is satisfactory) in the center well so that the top of the roll projects a millimeter or two above the rim of the center well; the alkali, being absorbed on the paper roll, thus projects into the gas space of the vessel and is better able to absorb CO_2.

(c) PREPARATION OF MANOMETER. When all the vessels are ready, mount each one on its manometer, using a small amount of suitable stopcock grease,[30] and attaching the vessels firmly to the manometer shanks by small springs or wound rubber bands. Fill each vessel containing tissue with oxygen by passing a slow stream of the washed gas from a tank[31] through the vessel, attaching the rubber tube delivering the gas to the top of the manometer so that the gas passes through the manometer stopcock and capillaries into the vessel and out the opened side bulb of the vessel. A few minutes' passage is sufficient for each vessel; all the vessels can be gassed at once if a multiple manifold attached to the tops of all the manometers is used. It is not necessary to gas the thermobarometer vessel.

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[28] Ringer's solution may be prepared as follows: to 960 ml. of 0.154 M NaCl solution add 20 ml. of 0.154 M KCl solution and 20 ml. of 0.11 M $CaCl_2$ solution.

[29] The usual medium is Ringer-phosphate. To 10 volumes of Ringer's solution add 1 volume of M/15 phosphate buffer, pH 7.4. For preparation of phosphate buffer, see Chapter 1. If glucose is desired in the medium, a level of 200 mg. per 100 ml. may be established by adding 2 ml. of 10 per cent glucose solution to 100 ml. of medium. The presence of glucose in the medium does not affect the oxygen consumption of liver slices, but is necessary with many other tissues.

[30] "Cello-Seal," obtainable from the Fisher Scientific Co., New York, is quite satisfactory. Ordinary petroleum jelly or anhydrous lanolin may also be used, but since these

When the vessel is filled with oxygen, turn the stopcock at the top of the manometer so as to close off the manometer and divert the flow of gas to the outside air; at the same time, have the greased plug for the side bulb ready, and as soon as the gas flow has been diverted, insert this plug and fasten it securely. Continue in this way with each of the other vessels being gassed.

Place the manometers one by one on the constant-temperature bath, which should be at 38° C. As each vessel enters the thermostat, the temperature rise will cause expansion of the enclosed gas and the manometer fluid will rise. Release the excess pressure by momentarily opening the manometer stopcock to the air and then closing it. This operation may have to be repeated. The thermobarometer is placed on the bath with the manometer stopcock open.

Start the shaking device and shake vessels and manometers for 10 minutes; this is usually sufficient to bring about temperature and pressure equilibrium. During this time, adjust the manometer fluid so that the level in the closed limb is approximately at the calibration setting (usually the 150-mm. point on the scale). Since the pressure change to be expected during the experiment will be negative, the level of manometer fluid in the open limb should be as high as possible, to permit maximum range of readings. It may be necessary here also to release the pressure within a vessel momentarily to permit the indicated adjustment of manometer fluid level in each limb. During the preliminary equilibrium period, close the thermobarometer also; the level of manometer fluid should be about the same in each limb here.

When measurements are to be begun, stop the shaking device, note the time, and immediately set the manometer fluid level in the inner (closed) limb of the first manometer at exactly 150 mm. Record the fluid level in the open limb, to the nearest mm. Now read the thermobarometer in the same way. For each vessel reading there must be a thermobarometer reading unless temperature and atmospheric pressure remain sufficiently constant so that one thermobarometer reading suffices for all the vessels; this is rarely true.

Continue with the reading of the second manometer in the same way, until all of the manometers containing tissue have been read. When this has been done, start the shaking device. Repeat the readings as above, at suitable time intervals, usually 10 or 15 minutes, for the duration of the experimental period.

CALCULATION. Subtract each reading for a vessel from the previous reading, and from this subtract algebraically the change in thermobarometer reading during the same time, to obtain the net change in pressure for the time interval covered by the readings. Multiply the pressure change, *h*, by the vessel constant for oxygen under

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become somewhat soft at 38° C., it usually is necessary to tighten the vessels on the manometers at least once during the preliminary incubation period when they are used.

[31] Tanks containing oxygen and other gas mixtures mentioned here may be obtained from the Ohio Chemical Co., New York City.

the particular experimental conditions, k_{O_2}, to obtain the oxygen consumption in microliters. Multiply this by $\frac{60}{t}$, where t is the time in minutes, and divide the result by the dry weight of the tissue, in mg., to obtain the Q_{O_2} value for the tissue.

$$h \times k_O = X_O \tag{1}$$

$$X_{O_2} \times \frac{60}{t} \times \frac{1}{\text{dry weight}} = Q_{O_2} \tag{2}$$

Example. The protocols of a typical experiment are illustrated as follows:

READINGS

Time (Minutes)	*Manometer No. 1*	*Thermo-barometer*	*Net Change*
0	282	151	
15	260	153	
	−22	2	−24
30	238	150	
	−22	−3	−19
45	211	146	
	−27	−4	−23
60	190	146	
	−21	0	−21
			−87 for 1 hr.

$h = -87$
$k_{O_2} = 1.43$ at 2 ml. V_F
$X_{O_2} = 1.43 \times -87 = -124\ \mu$l.
Wet weight of tissue = 98 mg.
% solids in tissue = 24.3
Dry weight of tissue = $98 \times 0.243 = 24$ mg.
$Q_{O_2} = -124 \times {}^{60}\!/_{60} \times {}^{1}\!/_{24} = -5.2$

The per cent of solids in the slices is obtained from the weight before and after drying overnight in an oven at 100° C. The water content of tissue slices stored in Ringer's solution is not necessarily the same as that of the original fresh tissue. Many of the data in the literature are based upon dry weights obtained by removing the slices from a vessel at the end of the experiment, draining, drying, and weighing. Results by this method usually are considerably higher than those obtained on the basis of the "initial" dry weight as described here, and may be seriously in error, particularly if (as usually happens), the slices disintegrate to some extent during an experiment. Results are occasionally reported on some basis other than the dry weight, such as in terms of milligrams of protein nitrogen, etc. The dry-weight procedure is most commonly followed.

With vessel constants of approximately 1.5, and a manometer scale length of 300 mm., it usually is possible to measure the absorption of about 400 μl. of oxygen without resetting the manometer. If it is wished to continue measurements beyond this point, the manometer may be reset by opening the manometer stopcock momentarily to admit a little air, closing it, and then adjusting the fluid level in the closed limb to the 150-mm. mark again, and reading the new level of fluid in the open limb against the thermobarometer as usual, continuing with the readings as described in the procedure above. If at the end of an experimental period the fluid level in the open limb is below the graduated range when the manometer is set at 150 mm., and it is desired to obtain a reading without resetting the manometer, set the manometer level at two successive points above the 150 mark—e.g., at 170 and at 160—make each reading quickly, and extrapolate to the reading at 150, since the change in reading between 160 and 150 will be the same as that between 170 and 160.

3. *Measurement of Anaerobic Glycolysis by Tissues.* Prepare the tissue as described under Exp. 2, but use Ringer-bicarbonate solution containing added glucose as the medium[32] and do not put any alkali into the center wells. After attaching the vessels to the manometers, pass a stream of washed nitrogen gas containing 5 per cent carbon dioxide[33] through the vessels for 20 to 30 minutes at room temperature to completely displace any oxygen present. During the gassing period run the manometer fluid up and down once or twice to replace the air in the manometer capillary by the gas mixture, and shake the vessels occasionally to aid in displacing any air bubbles trapped in the medium. In turning the gas off and closing the vessels, take suitable precautions against the possible diffusion of room air into the system in the process. Vessels with bored plugs in the side bulbs, which can be left in place but clear for the passage of gas during the gassing period, and then closed by a quarter-turn, are much to be preferred over the type having solid side-bulb plugs.

The rise in pressure caused by immersing the vessels in the thermostat at 38° is released as described under Exp. 2, again with precautions against allowing air to reënter the system. Since the readings will always be positive in this type of experiment (i.e., the pressure will increase owing to decomposition of bicarbonate by the acid produced), set the manometer fluid level in the open limb at the bottom of the scale rather than at the top. This is easily done by forcing up the manometer fluid in the closed limb to about 200 mm., opening the stopcock cautiously to release most (but not all) of the excess pressure; then closing the stopcock and lowering the manometer fluid level to the 150 mark on the closed limb. If this is done properly, the fluid level in the open limb will be low down on the scale at this point.

Equilibrate by shaking for 10 minutes; then start readings, making them against a thermobarometer (which need not be gassed) just as described for the measurement of oxygen consumption in Exp. 2. Read at suitable intervals for the desired length of time.

CALCULATION. Subtract each reading from the next one, and subtract algebraically from this the change in thermobarometer reading during the same period, as for Exp. 2, to obtain the net increase in pressure due to acid production. Multiply by the vessel constant for CO_2 at the fluid volume used, k_{CO_2}, to obtain the number of μl. of CO_2 evolved as the result of acid production. Express results in terms of $Q_G^{N_2}$, which is defined as the number of microliters of CO_2 equivalent to the acid produced by 1 mg. dry weight of tissue in one hour, under anaerobic conditions.

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[32] To 5 volumes of Ringer's solution add 1 volume of 0.15 M $NaHCO_3$ solution. Bubble the nitrogen (95 per cent)–CO_2 (5 per cent) gas mixture through this solution for about 15 minutes before using, to bring to pH 7.4. A glucose concentration of approximately 200 mg. per 100 ml. may be established by adding 2 ml. of 10 per cent glucose solution to each 100 ml. of medium.

[33] Tanks containing 95 per cent nitrogen–5 per cent carbon dioxide gas mixture may be obtained from the Ohio Chemical Co., New York City. The gas mixture may be freed from the traces of oxygen usually present by passing through a heated tube filled with metallic copper turnings. Pass the dried gas through a wash bottle containing water to saturate with water vapor before entering the vessels.

With certain tissues anaerobic glycolysis proceeds linearly for hours, provided sufficient glucose is present; with other tissues the rate of glycolysis is independent of the presence of glucose (and hence is presumably at the expense of glycogen) and may show a continual decrease with time. In this latter instance, the Q values represent the average for an hour, or may be computed on the basis of shorter experimental periods.

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13

Salivary Digestion

Digestion in General. The greater part of the food elements in the diet require special treatment to render them capable of absorption and utilization by the body. Water, glucose, and certain inorganic salts and vitamins are exceptions, but the proteins, fats, and carbohydrates, as well as other substances, must be split up into simple components as monosaccharides, glycerol, fatty acids, amino acids, etc. The necessary changes are chiefly hydrolytic in character and involve especially the action of enzymes found in the different parts of the gastrointestinal tract. Because of the variations in the quantity and character of food ingested and the variety of changes that must be brought about, the different parts of the gastrointestinal tract must show a considerable power of adaptation and coördination. In this, both hormone and nervous mechanisms are concerned.

Certain changes similar to those occurring in digestion may take place in foods prior to ingestion. In the ripening of certain fruits, such as the banana, starch is changed to dextrin and maltose through the action of amylase. Meats on storage undergo some self-digestion or autolysis. This process may be hastened for commercial purposes ("tenderizing") by the application of proteolytic enzyme preparations to meat cuts. In the cooking of foods connective-tissue fibers are gelatinized and starch granules are broken up and some dextrinization of starchy foods occurs. Cooking also increases the palatability of foods and in this way promotes the secretion of digestive juices.

Secretion of Saliva. The saliva is secreted by three pairs of glands—parotid, submaxillary, and sublingual—reinforced by numerous small glands called buccal glands. These secretions vary in character. Thus in man the parotid saliva is watery and has a high digestive power. The secretions of the other glands are higher in mucin and more viscid. Ordinary saliva is a mixture of the secretions of these glands and shows considerable variations in composition in different individuals, and in the same individual at different times.

The secretion of saliva is governed by a set of nerve fibers cerebral in origin, together with fibers from the sympathetic nervous system. No hormone mechanism in salivary secretion is known. Ordinarily the secretion of saliva is the result of a reflex stimulation of the secretory nerves through a center in the medulla oblongata. Psychic stimuli, brought about by such influences as the thought of food, pass from the higher nerve centers to the secretory center and also give rise to secretion. The results of Pavlov on dogs show it to be rather difficult in certain cases to differentiate between the two types of stimuli. Pavlov found that dropping several pebbles into a dog's mouth caused the flow of but one or two drops of saliva, but sand in the mouth induced a copious flow of a thin watery saliva. Ice water caused no secretion, but acid or bitter solutions which the animal wished to reject caused a free flow of saliva. Dry food caused the secretion of a watery saliva; meat led to the flow of a more slimy secretion, such as would aid in the lubrication of this food for swallowing. Drawing the attention of the animal to these foods, without actually giving them to him, gave rise to similar secretions. Thus, also, the pretense of throwing sand into the mouth of the dog gave rise to a profuse watery secretion.

The amount of saliva secreted by an adult in 24 hours has been variously placed, as the result of experiment and observation, between 1000 and 1500 ml., the exact amount depending, among other conditions, upon the character of the food. In the absence of obvious external stimuli, the rate of salivary secretion in the adult appears to be between 0.1 ml. and 0.9 ml. per minute.

Composition of Saliva. Salivary composition depends on many factors: stimulation, diet, age, time of day, disease, etc. To insure reproducible, representative samples for analysis, the conditions for collection should include (*a*) a definite physiological, postabsorptive state (before breakfast), (*b*) no brushing of the teeth, rinsing of the mouth, or smoking prior to collection, (*c*) about a two-hour interval between arising and collection, including a 15-minute rest period immediately preceding collection. Stimulation yields a relatively more dilute saliva, but the difference diminishes as collection is prolonged.

Ordinarily saliva varies from weakly alkaline to weakly acid, the pH ranging approximately from 6.0 to 7.9 with an optimum pH of 6.6. There is evidence that normal individuals under 20 and without dental defects secrete a saliva with a pH between 7.0 and 7.2. No absolute correlation has been shown between salivary acidity and caries or other dental disorders. However, lower pH values occur more frequently among caries-susceptible individuals. There is evidence that increased acidity of saliva is a late rather than early manifestation of the caries syndrome as compared with changes in the level of other salivary constituents. Dental erosion is usually accompanied by greatly increased total salivary acidity. The acid secretion from the gingival crevice and the marginal gingiva tends to dissolve enamel in the regions already predisposed as a result of unfavorable metabolic conditions.

Saliva is a dilute secretion having an average specific gravity of 1.007 with about 0.7 per cent of solid matter, about 0.5 per cent being organic and 0.2 per cent inorganic. Of the organic matter about 0.4 per cent is protein, chiefly mucin, with small amounts of albumin and globulin. Other organic constituents are enzymes (chiefly salivary amylase), urea, uric acid, cholesterol, vitamins, and phospholipide. Average values have been reported of total nonprotein nitrogen 13 mg., urea plus ammonia nitrogen 11 mg., and uric acid 1.5 mg. per 100 ml. These amounts averaged 37 per cent, 76 per cent, and 40 per cent, respectively, of the corresponding constituents in blood. Analyses of the saliva for these constituents possess a certain clinical value in the study of nephritis but are little used for this purpose. Normal saliva contains no glucose. Increases in cholesterol and in lipide phosphorus have been noted in certain dental disorders.

The normal composition of saliva is shown in the accompanying table.

COMPOSITION OF NORMAL SALIVA*

Constituent	*Normal Range*	
Acidity (pH)	6.0 – 7.9	
Titratable alkalinity (as 0.02 N HCl)	90.0 – 190.0	ml. per 100 ml.
Ammonia N	2.0 – 10.0	mg. per 100 ml.
Calcium, total, as Ca	4.0 – 8.0	mg. per 100 ml.
Inorganic phosphate, as P	10.0 – 25.0	mg. per 100 ml.
Chloride, as Cl	30.0 – 60.0	mg. per 100 ml.
Carbonate, as CO_2	20.0 – 45.0	ml. per 100 ml.
Protein	200.0 – 400.0	mg. per 100 ml.
Cholesterol	2.5 – 9.0	mg. per 100 ml.
Lipide P	0.05 – 0.20	mg. per 100 ml.

* The values here given are based on saliva collected in a postabsorptive state, and (with the exception of those for cholesterol and lipide P) by stimulation. Possible relationship to caries may be found in the following constituents: ammonia, calcium and phosphate (especially the adsorbable fractions), carbonate, cholesterol, and lipide P. Acknowledgment is made to Dr. Frances Krasnow for the data from which this table was compiled.

Potassium thiocyanate, KSCN, is also generally present in the saliva to the extent of several milligrams per milliliter. The significance of thiocyanate in the saliva is not known; it may possibly come from the ingested cyanides present in certain fruits and in tobacco smoke and from the breaking down of protein material. *Apoerythein,* a protein fraction which protects vitamin B_{12} from digestive destruction is also present in saliva. This may be identical with the *intrinsic factor of Castle.*

Dam and his associates[1] have shown the following average content of vitamins in saliva (gamma (γ) per ml.): Thiamine 0.007, riboflavin 0.05, niacin 0.03, pyridoxine 0.6, pantothenic acid (as Ca salt) 0.08, folic acid 0.0001, biotin 0.0008, ascorbic acid 2.4, vitamin K (as menadione) 0.015.

The so-called tartar formation on the teeth is composed almost entirely of calcium and magnesium phosphates with some calcium carbonate, mucin, epithelial cells, and organic debris derived from the food. The calcium salts are held in solution as acid salts, and are probably precipitated by alkalinity caused by ammonia formation through bacterial action or through loss of CO_2 from the saliva. The various organic substances just mentioned are carried down in the precipitation of the calcium salts. There is evidence of increased salivary calcium in individuals suffering from excessive tartar deposition.

So-called salivary calculi may form in the gland or in the duct. Their composition is similar to that of tartar. This condition is known as *sialolithiasis.*

Salivary Amylase. The principal enzyme of the saliva is known as salivary amylase. The name ptyalin, formerly used for this enzyme, is now obsolete. Salivary amylase is an amylolytic enzyme. The enzyme catalyzes the hydrolytic splitting of starch, glycogen, and the dextrins into simpler molecules, the process being a progressive one with the disaccharide maltose as the ultimate end product.[1]

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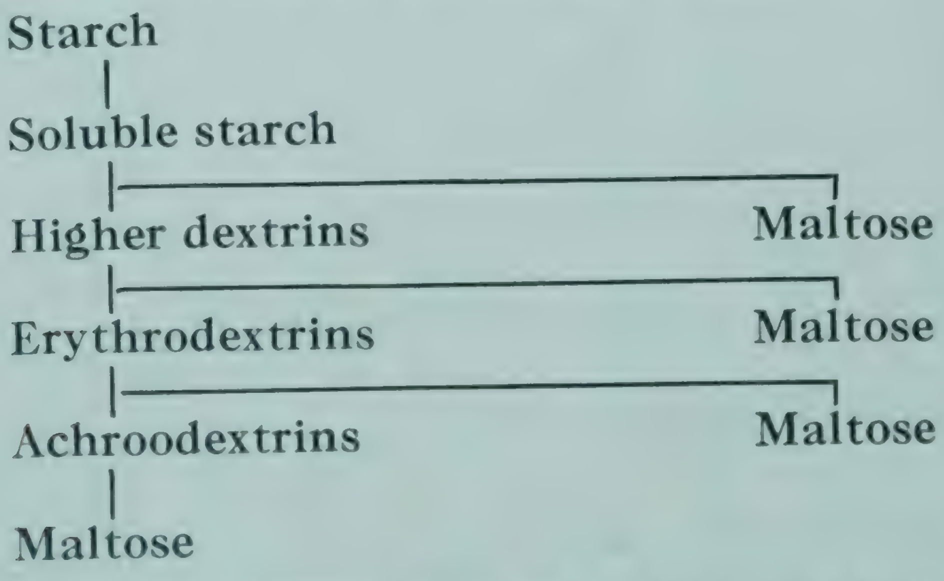
[1] Dam et al.: *Intern. Z. Vitaminforsch.*, **20**, 234 (1948).

The opinion has been confirmed that the salivary amylase activity varies with the composition of the diet and is highest on a diet which is predominantly carbohydrate.[2]

The first product of the action of the amylase on starch is soluble starch, whose formation is indicated by the disappearance of the opalescence of the starch solution. Soluble starch gives a blue color with iodine; its formation from starch is not associated with the production of free reducing sugar (maltose). Further action of amylase on starch apparently consists largely in the hydrolytic splitting of the second glycoside linkage from a free end of the long chain (straight or branched) of glucose residues which make up the starch molecule. This action produces the disaccharide maltose and a series of smaller polysaccharide molecules which are relatively ill-defined and which are known as the dextrins.

The various dextrins differ in molecular size and complexity, depending upon the extent of amylase action. The higher members of the series resemble starch in giving a blue or purple color with iodine; as the molecule becomes smaller by the splitting off of maltose, a red color is given with iodine (*erythrodextrins*), and the lower members of the series give no color at all with iodine (*achroodextrins*). Thus during the action of salivary α-amylase on starch, free maltose is produced almost immediately and progressively increases in amount; at the same time the color reaction with iodine changes from blue through red to colorless. Both the increase in reducing sugar and the change in the iodine color reaction are used in following the course of action of amylase on starch. The changes described above may be represented as follows:



These steps in the breakdown of straight and branched glucose chains in amylose and amylopectin are illustrated in Figs. 83 and 84 respectively.

It must be borne in mind, however, that our knowledge of the course of starch hydrolysis by amylase is incomplete. This is due to the complicated nature of both starch (see Chapter 2) and amylase. Most amylases appear to be mixtures of two enzymes, α- and β-amylase, which are said to produce respectively α- and β-maltoses on starch hydrolysis. According to one study on malt amylase, starch is completely hydrolyzed to maltose by α- + β-amylase + a complement present with these in malt. Without the complement, 80 per cent of the starch may be split, leaving 20 per cent of residual dextrin with slight reducing power. α-Amylase alone leaves about 60 per cent of dextrins giving no color with iodine, while β-amylase alone leaves about 40 per cent of dextrins giving a blue color with iodine. The amylase of saliva is chiefly α-amylase. Both amylases are found in malt extract. Malt extract kept for several days at 0° C. and at pH 3.6 contains only β-amylase. α-Amylase is obtained by extraction of ungerminated barley with water.

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[2] Squires: *J. Physiol.*, **119**, 153 (1953).

Salivary amylase acts in alkaline, neutral, or faintly acid solutions. The optimum acidity is pH 6.6. Amylase is destroyed at acidities greater than pH 4 or at concentrations of free HCl greater than 0.0005 per cent. By sufficiently increasing the alkalinity of the saliva, the action of the salivary amylase is inhibited. Salivary amylase has been crystallized.[3]

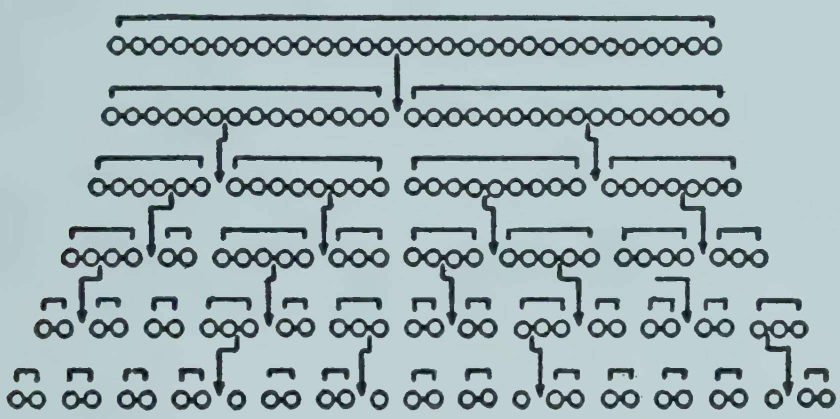

Fig. 83. Action of α-Amylase on Amylose: (○) Glucose Unit; (-○-○-) α-1,4-Glucosidic Linkage; (↓) α-Amylase Action.

P. Bernfeld: *Adv. Enzymol.*, **12**, 379 (1951).

Electrolytes have an important influence upon the action of amylases. For example, Rockwood has shown that Cl, Br, and NO_3 ions have a pronounced stimulating action upon salivary amylase. Removal of chlorides from saliva by dialysis renders the amylase inactive. Amino acids, particularly asparagine, have an accelerating action. Salts of the heavy metals such as silver and mercury inhibit because they combine with the enzyme, which is apparently of a protein nature.

Because of its sensitivity to acid, salivary amylase ceases to act in the stomach as soon as the gastric contents show throughout the presence of free hydrochloric acid. Since, however, the amylolytic activity of human saliva is very great, an appreciable digestion of starch may occur during the period of mastication and swallowing. Furthermore, the food which is swallowed is not immediately mixed in its entirety with gastric juice and the protein of the food has a certain binding power for free acid, so that a certain interval may intervene between the entrance of the food into the stomach and the destruction of the amylase. This period varies much in different animals and depends also on the size and character of the meal. In certain experiments on normal men Bergeim found that salivary digestion might continue for 15 to 30 minutes, and that in meals with bread and mashed potatoes there was a conversion of the starch to maltose of about 60 and 75 per cent, respectively.

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[3] Meyer, et al.: *Helv. chim. Acta*, **31**, 2158 (1948).

The digestion of the starch of foods decreases their bulk, and for this reason, and because starch has some inhibitory action on pepsin, salivary digestion may have some favorable effect on protein digestion in the stomach. The action of salivary amylase is not, however, essential, since the pancreatic juice contains a powerful amylase.

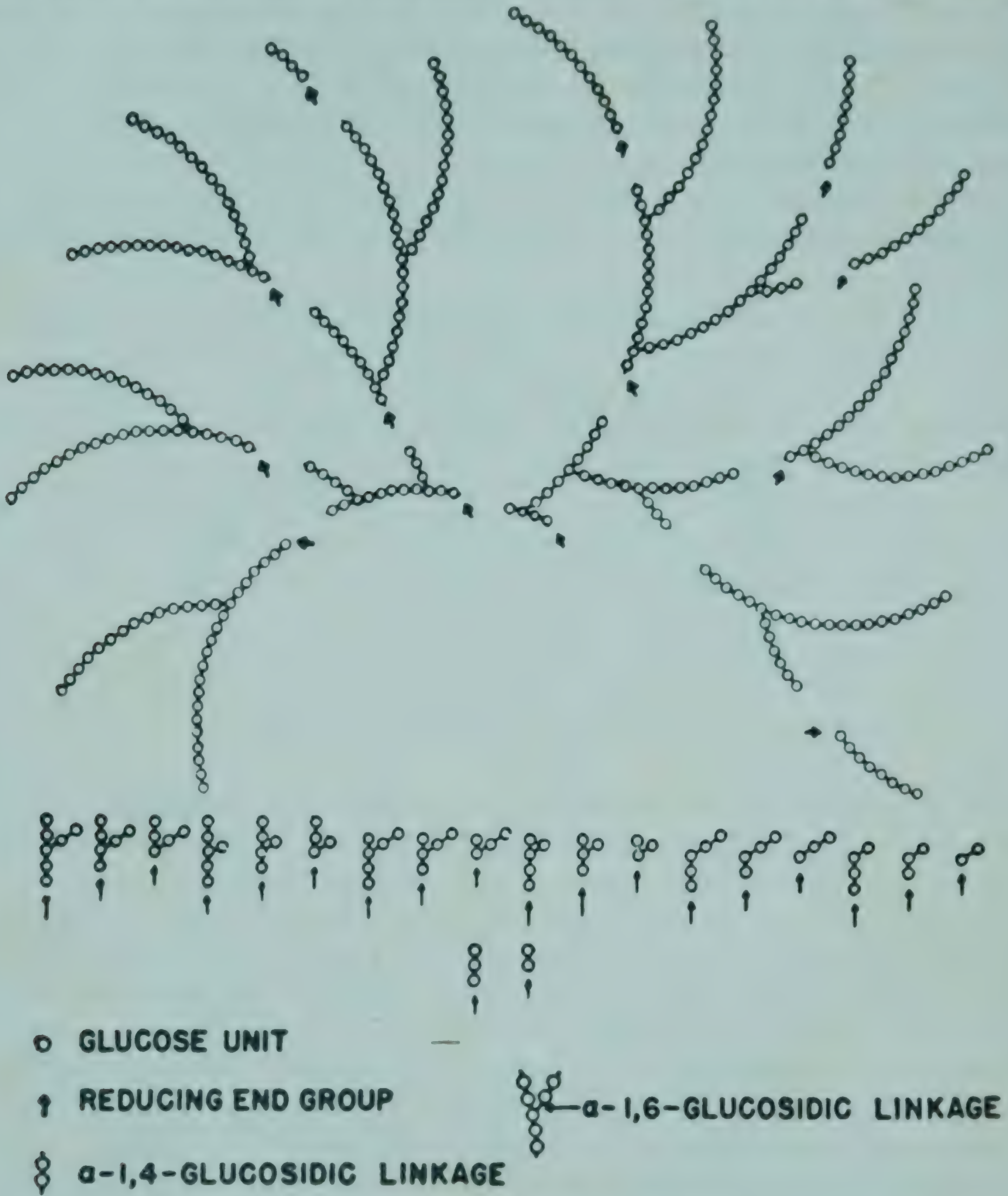

FIG. 84. ACTION OF α-AMYLASE ON AMYLOPECTIN.

Upper portion: Formation of iodine-reacting dextrins of medium molecular weight. *Lower portion:* Possible structures of limit dextrins (heptasaccharides, hexasaccharides, etc.).

P. Bernfeld: *Adv. Enzymol.*, **12**, 379 (1951).

The saliva of rodents is amylolytic. Hog saliva contains amylase, but much less than human saliva. The saliva of carnivorous animals is free from amylase, as is also that of herbivorous animals.

Maltase is found in traces in saliva. Maltase splits maltose into glucose but the amount of such digestion in the mouth is very slight. It is claimed that dipeptide- and tripeptide-splitting enzymes are present in saliva. This action in at least some cases is due to bacteria and in any case is not of digestive importance.

Mucin. Mucin gives saliva its viscosity. It is a glycoprotein, insoluble in water or dilute acid but soluble in dilute alkali. When precipitated, as upon the teeth, mucin forms with alkali a slippery mass which dissolves but slowly. It is therefore removed from the teeth with difficulty and may furnish a nucleus for the deposit of other substances. Mucin may be precipitated from saliva by dilute acid or by alcohol. It is an acid substance existing in saliva as the potassium salt. It gives the usual protein color reactions, but is not coagulated by heat in neutral solutions. It is precipitated by saturating with ammonium sulfate. On hydrolysis it yields besides protein a mucoitin sulfuric acid which on further decomposition gives sulfuric acid, acetic acid, glucuronic acid, and glucosamine.

Microscopical examination of saliva reveals epithelial cells, salivary corpuscles (white blood cells?), mucus, food debris, and numerous microorganisms from the true bacteria, higher bacteria, fungi, and protozoa groups. Pus cells and red blood cells may be evident in pathological conditions of the mouth.

EXPERIMENTS ON SALIVA

A satisfactory method of obtaining the saliva necessary for the experiments which follow is to chew a small piece of pure paraffin wax, thus stimulating the flow of the secretion, which may be collected in a small beaker. It must be remembered in this connection that paraffin-stimulated saliva is quite different in some respects (e.g., pH) from ordinary saliva. Filtered saliva should be used in every experiment except the microscopical examination and the quantitative determination of amylase activity.

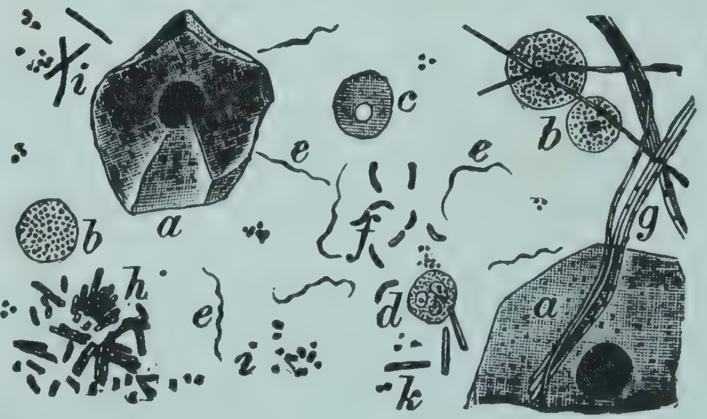

FIG. 85. MICROSCOPIC CONSTITUENTS OF SALIVA.

a Epithelial cells, *b* salivary corpuscles, *c* fat drops, *d* leukocytes, *e*, *f*, *g* bacteria, *h*, *i*, *k* fission-fungi.

1. *Microscopical Examination.* Examine a drop of unfiltered saliva microscopically, after staining with methylene blue, and compare with Fig. 85.

2. *Reaction.* Test the reaction to litmus or other suitable indicator paper. Estimate the approximate pH of the saliva. Measure 2 ml. of fresh saliva into a small test tube. Add 10 drops of an indicator solution suitable for measuring pH at the estimated pH of the saliva.[4] Compare with 2-ml. portions of pH standard solutions[4] treated with the same amount of indicator in similar tubes. The standards should differ by

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[4] See Chapter 1 for the preparation and use of indicators, standards, and the comparator block.

0.2 pH unit.[5] Determine the pH of the saliva. The comparison is best made in a comparator block, with a tube containing saliva only placed behind the standard, and a tube of plain water behind the saliva-indicator tube.

3. Test for Mucin. To a small amount of saliva in a test tube add 1 to 2 drops of dilute acetic acid. Mucin is precipitated.

4. Biuret Test. Render a little saliva alkaline with an equal volume of NaOH and add a few drops of a very dilute (2 to 5 drops in a test tube of water) copper sulfate solution. The formation of a purplish-violet color is due to mucin. This reaction is given by protein material and simply indicates that mucin is a protein.

5. Preparation of Mucin. Pour 25 ml. of saliva into 100 ml. of 95 per cent alcohol, stirring constantly. Cover the vessel and allow the precipitate to stand at least 12 hours. Pour off the supernatant liquid, collect the precipitate on a filter, and wash it, in turn, with alcohol and ether. Finally dry the precipitate, remove it from the paper, and make the following tests on the mucin: (*a*) Test its solubility in water, dilute acid, and dilute alkali; (*b*) Millon's reaction; (*c*) dissolve a small amount in NaOH, and try the biuret test on the solution; (*d*) boil the remainder, with 10 to 25 ml. of water to which 5 ml. of dilute HCl has been added, until the solution becomes brownish. Cool, render alkaline with solid sodium carbonate, and test by Benedict's solution. Reduction should take place. Why? Does the unhydrolyzed mucin reduce Benedict's solution?

Mucin may also be prepared from salivary glands.

6. Viscosity Test. Place filter papers in two funnels, and to each add an equal quantity of starch paste (5 ml.). Add a few drops of saliva to one lot of paste and an equivalent amount of water to the other. Note the progress of filtration in each case. Why does one solution filter more rapidly than the other?

7. Test for Nitrites. Add 1 to 2 drops of dilute H_2SO_4 to a little saliva and stir thoroughly. Now add a few drops of a freshly prepared potassium iodide solution and some starch paste. Nitrous acid is formed which liberates iodine, causing the formation of a blue color with the starch.

8. Thiocyanate Test: Solera's Reaction. This test depends upon the liberation of iodine through the action of thiocyanate upon iodic acid. Moisten a strip of starch paste–iodic acid test paper[6] with a little saliva. If thiocyanate is present the test paper will assume a blue color, owing to the liberation of iodine and the subsequent formation of the so-called iodide of starch.

9. Digestion of Starch Paste. To 25 ml. of starch paste in a small beaker, add 5 drops of saliva and stir thoroughly. At intervals of a minute remove a drop of the solution to one of the depressions in a test tablet and test by the iodine test. At the same time add 3 drops of the mixture to one of a series of test tubes set up with 5-ml. portions of Benedict's reagent. The opalescence of the starch solution should soon disappear, indicating the formation of soluble starch which gives a blue color with iodine. The soluble starch should soon be transformed into erythrodextrin which gives a red color with iodine, and this in turn should pass into achroodextrin which gives no color with iodine. This is called the achromic point. When this point is reached, complete the Benedict tests by placing all the tubes in a boiling water bath for 3 minutes, and note the degree of reduction in each tube. Tabulate your results with the iodine and Benedict tests in parallel columns. Also perform a phenylhydrazine test for maltose (the osazone crystals may not have the typical appearance of maltosazone). A positive Benedict test may be obtained while the solution still reacts red with iodine, inasmuch as some maltose is formed from the soluble starch coincidently with the formation of the erythrodextrin. How long did it take for a complete transformation of the starch? Saliva from different individuals may vary markedly in amylolytic power. For a graphic representation of the above changes see p. 352.

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[5] To obtain a closer approximation of the reaction of saliva in the mouth, draw the sample into a pipet directly from the mouth (without previous chewing of paraffin), and dilute with the indicator in the test tube, under a thick layer of mineral oil. (Krasnow, Oblatt, and Kaplan: *J. Dental Research*, 15, 367 (1936).)

[6] See Appendix.

10. Separation of the Products of Salivary Digestion. To 25 ml. of 1 per cent starch paste in a small beaker add 1 ml. of saliva and stir thoroughly. At intervals of one minute test a drop of the mixture by the iodine test. If the blue color persists after five minutes add another 1 ml. of saliva. When the mixture reacts red with iodine, indicating that erythrodextrin has been formed, add 100 ml. of 95 per cent alcohol. Allow to stand until the white precipitate has settled. Filter, evaporate the filtrate to dryness on a water bath, dissolve the residue in 5 to 10 ml. of water, and try Benedict's test and the phenylhydrazine reaction. On the dextrin precipitate try the iodine test.

11. Influence of Temperature. Into each of four tubes place about 5 ml. of 0.2 per cent starch paste. Immerse one tube in a beaker containing crushed ice, keep a second at room temperature, and place a third in the incubator or the water bath at 40° C. (If the temperature of the bath or incubator is allowed to rise to 70° C. or over the enzyme is destroyed and no digestion takes place.) Now add to the contents of each of these three tubes 5 drops of saliva and shake well; to the contents of the fourth tube add 5 drops of boiled saliva. Test frequently by the iodine test, using the test tablet, and note in which tube the most rapid digestion occurs. Explain the results.

12. Estimation of Amylase in Saliva. Pipet exactly 1 ml. of unfiltered saliva into a 100-ml. cylinder. Dilute to the 100-ml. mark, and mix well. Pipet 5 ml. of 1 per cent soluble starch into a test tube. Add 2 ml. of 1 per cent NaCl solution and 2 ml. of a buffer solution of pH 6.6[7] and put in a water bath maintained at 38° C. Prepare a series of 10 test tubes, each containing 2 ml. of a light yellow iodine solution. Now add 1 ml. of diluted saliva to the starch mixture and return to the bath at once. Record the time of this addition. At the end of each minute of digestion, with a pipet remove 2 drops of the mixture and add to one of the tubes of iodine solution. Record the time when no change in color appears in the iodine solution (achromic point). If this time is less than 5 or more than 20 minutes, repeat, using a different dilution of saliva such as will give a digestion time of about 10 minutes. Thus if 3 minutes are required, dilute 30 ml. of the original diluted saliva to 100 ml., and use 1 ml. of this for the test.

One unit of amylase may be considered to be the amount required to digest 5 ml. of 1 per cent soluble starch to the achromic point in 10 minutes under the conditions of the test. The number of units of amylase in 1 ml. of the saliva tested will equal 100 (or whatever the dilution of the original saliva might be) × 10 ÷ the number of minutes to the achromic point. From 100 to 150 units are frequently found.

13. Influence of NaCl on Salivary Amylase. Repeat the preceding experiment but replace the NaCl solution by water. A longer time should be required, showing that NaCl accelerates the action of salivary amylase. If saliva be dialyzed free from chlorides it becomes inactive.

14. Influence of pH on Salivary Amylase. In Exp. 12 the solution had a pH of 6.6. Replace the phosphate buffer of this experiment by another buffer of pH 5.8 and in a second experiment use a buffer of pH 8.0.[7] Which gives digestion in the shortest time and is hence nearest the optimum pH?

15. Excretion of Potassium Iodide. With the aid of a glass of water, ingest a small dose of potassium iodide (0.2 g.) contained in a gelatin capsule, quickly rinse out the mouth with water, and then test the saliva at once for iodine. This test should be negative. Make additional tests for iodine at two-minute intervals. The test for iodine is made as follows: Take 1 ml. of dilute $NaNO_2$ solution and 1 ml. of dilute H_2SO_4[8] in a test tube; add a little saliva directly from the mouth, and a small amount of starch paste. The formation of a blue color signifies that the potassium iodide is being excreted through the salivary glands. Note the length of time elapsing between the ingestion of the potassium iodide and the appearance of the first traces of the substance in the saliva. If convenient, the urine may also be tested at 15- or 30-minute intervals after ingestion of the iodide. The chemical reactions taking place in this experiment are indicated in the following equations:

$$\begin{array}{ll} (a) & 2NaNO_2 + H_2SO_4 \rightarrow 2HNO_2 + Na_2SO_4 \\ (b) & 2KI + H_2SO_4 \rightarrow 2HI + K_2SO_4 \\ (c) & 2HNO_2 + 2HI \rightarrow I_2 + 2H_2O + 2NO \end{array}$$

Inasmuch as iodide is absorbed from the stomach very slowly, if at all, but is very rapidly absorbed when it enters the intestine, the rapidity of appearance of the iodide in the saliva is an index of the rapidity with which the drug leaves the stomach, which depends on the motor activity of the stomach, the amount of food therein, etc. By drinking a glass of water with the iodide a more rapid result is obtained.

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[7] See Chapter 1.

[8] Instead of this mixture a few drops of HNO_3 possessing a yellowish or brownish color (containing HNO_2) may be employed.

14

Gastric Digestion

Following mastication the food is carried by peristaltic movements of the esophagus to the stomach. Here it undergoes further mechanical disintegration and chemical changes, primarily in the protein constituents. The food thus treated is in a better condition to be handled by the intestines, to which it is passed on in small portions at a time, and in which digestion is completed.

Spallanzani (1783) found that gastric juice dissolved meat, and so demonstrated the chemical nature of gastric digestion. He noted also that the juice was acid, but the nature of this acid and of the active agent pepsin was not demonstrated until later.

A great advance in our knowledge of gastric digestion, particularly in man, was made through the observations of Beaumont on his patient Alexis St. Martin, who in 1822, following a gunshot wound, was left with an opening from the stomach through the abdominal wall to the exterior. Through this fistula Beaumont found it possible to follow the course of gastric digestion with different foods and under varying conditions of health, and to obtain pure gastric juice for digestion experiments outside the body.

Pavlov extended our knowledge, particularly through the development of an operation by means of which he created in dogs a small stomach pouch separate from the main stomach, and opening to the exterior, so that the secretion in the small pouch could be studied without interfering with processes in the stomach proper. In this way the influence of different foods and of other factors on gastric secretion could be studied. The development of the small stomach tube which could be retained in place throughout the period of gastric digestion and allowed aspiration of the stomach contents at any time, has given additional information of physiological and clinical value.

Secretion of Gastric Juice. There is a slight continuous secretion of gastric juice into the empty stomach. As a result there is almost always present in the stomach before meals about 50 ml. of secretion which is called the residuum. Gastric secretion is governed by many factors. At least three phases are recognized.

(1) Psychic Phase. Following the presentation of food and before any food reaches the stomach, there is a psychic secretion of gastric juice. This is induced by the sight, taste, smell or thought of food. Ivy has called this the "cephalic phase."

(2) Gastric Phase. Following this, with the passage of food into the stomach, there occurs the gastric phase of gastric secretion due to the

local chemical action of such substances as protein digestion products, or meat extractives, and some mechanical action due to friction and distention of the stomach by food. A substance, *gastrin*, has been isolated from gastric mucosa (Edkins). When injected into the blood, this markedly stimulates gastric secretion. It is believed to be a hormone of gastric secretion, i.e., a substance liberated from the gastric mucosa in the presence of food and passing by way of the blood to the acid-secreting cells, stimulating them to action. *Histamine*, the decarboxylation product of the amino acid histidine is also a powerful stimulant of gastric secretion in addition to its well-known effect on blood pressure (capillary dilatation). The question whether or not gastrin is identical with histamine was unanswered for some years. They are now believed to be separate substances which possess similar action.[1] The chemical structure of histamine is shown on p. 1037. The structure of gastrin is not known. Clinical use is made of the secretagogue effect of histamine, alcohol, and caffeine.

(3) INTESTINAL PHASE. A third, intestinal phase of gastric secretion is brought about through the action of protein digestion products and other food substances in the intestines. Whether these substances act by liberating a hormone from the mucosa, or are absorbed and themselves act upon the gastric cells is not yet clear. Undigested fat in the intestine inhibits gastric secretion, apparently by liberating from the mucosa a hormone called enterogastrone which depresses the action of the gastric cells. This *chalone* also inhibits gastric movements and was for a time considered useful in the treatment of gastric ulcer. However, evidence of its ineffectiveness has accumulated. For example, Wollum and Pollard[2] found no significant alteration in the secretory or motor patterns from its use, and Bone[3] has reported that it has little or no value. Clinical research has indicated that the chemical substances called *Banthine*, *Pro-banthine*, and *Prantal* are useful in the treatment of ulcer (see p. 383). Urogastrone, a substance similar in action to enterogastrone and found in the urine, is apparently a metabolic derivative of enterogastrone.

Water has a stimulating action on gastric secretion, and the drinking of considerable water has been shown to improve the utilization of various foods. Nor has the drinking of water with meals by normal individuals been shown to be undesirable.[4] The influence of different foods on gastric secretion is discussed later.

The study of gastric secretion and the gastric mucosa in nutritional deficiencies has received some attention. Thus in canine blacktongue (the closest animal counterpart to human pellagra), a mild pallor of the gastric mucosa was observed consistently upon gastroscopic examination.[5] This was accompanied by anemia, loss of weight, and decreased muscular tone of the stomach wall. The tonus of the stomach returned to normal as early as six to seven days after the institution of therapy. There was no change in acid gastric secretion during the disease or following niacin therapy. The therapeutic effect of thiamine was also negative. Shapiro and his co-workers[6] found atrophic gastritis to be somewhat more common in nutritional deficiencies, than in a control group of ten other patients. However, no significant changes in the gastric mucosa were observed after treatment with large doses of thiamine, niacin, riboflavin, pantothenic acid, *p*-aminobenzoic acid, and vitamin A. In two cases the atrophic changes disappeared after choline chloride therapy.

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[1] Friedman and King: *Federation Proc.*, **6**, 107 (1947).

[2] Wollum and Pollard: *Gastroenterology*, **17**, 535 (1951).

[3] Bone *et al.*: *Gastroenterology*, **17**, 35 (1951).

[4] Hawk: *Endocrinology and Metabolism*, Barker, Hoskins, and Mosenthal, New York, D. Appleton & Co., 1924, Table 3, p. 275.

[5] Layne and Carey: *Gastroenterology*, **2**, 133 (1944).

Composition of Gastric Juice. Normal gastric juice is a thin, light-colored fluid which is acid in reaction and has a specific gravity averaging about 1.007. It contains about 0.5 per cent of solid matter which is made up principally of sodium chloride, potassium chloride, earthy phosphates, mucin, and the enzymes pepsin and gastric lipase. The acidity of the gastric juice is due to *free* hydrochloric acid. The gastric juice is a composite secretion from at least three different types of cells in the gastric mucosa; these are (1) the parietal cells, (2) the chief cells, and (3) the mucous cells. There is good evidence that the parietal cells furnish the hydrochloric acid of gastric juice, the chief cells supply pepsin and possibly other enzymes, and the mucous cells secrete mucin. Babkin[7] claims that the secretory activity of the various types of gland cells should not be considered en masse but rather that "various nerves . . . or chemical agents stimulate or inhibit each set of secretory elements separately." This view is also accepted by others.[8]

Apoerythein, a protein fraction, also occurs in gastric juice.[9] It has been suggested that this substance may be identical with the *intrinsic factor of Castle*, which protects vitamin B_{12} (the extrinsic factor) from digestive destruction. Pernicious anemia is characterized by a deficiency of the intrinsic factor. Vitamin B_{12} is relatively ineffective when given orally to such patients, but is highly effective when administered parenterally.

It is believed that the parietal-cell secretion is essentially an isotonic solution consisting largely of hydrochloric acid (about 160 milliequivalents per liter) and potassium chloride (about 7 milliequivalents per liter). The acidity of the parietal-cell secretion corresponds therefore to a solution 0.16 N in hydrochloric acid, or containing 0.5 to 0.6 per cent hydrochloric acid. This maximal acidity, which is apparently constant and independent of the rate of secretion, is lowered somewhat as soon as the parietal-cell secretion becomes admixed with the slightly alkaline secretions from the chief cells and mucous cells. These latter secretions contain a high concentration of neutral chlorides as a result of which the acidity is reduced to a greater extent than is total chloride content. The acidity may also be lowered by regurgitation of alkaline fluid from the intestine and by ingested food; so that the actual acidity of gastric juice as collected usually varies between 0.05 and 0.1 N (0.18 to 0.36 per cent hydrochloric acid). The acidity of the gastric juice is usually expressed in terms of the number of milliliters of 0.1 N sodium hydroxide required to neutralize 100 ml. of gastric juice; this is obviously equivalent to the number of milliliters of 0.1 N hydrochloric acid present in 100 ml. of gastric juice, which corresponds numerically with the concentration of acid expressed in terms of milliequivalents per liter. In clinical practice this value is sometimes called the degree of acidity; thus a gastric juice containing 60 milliequivalents of acid per liter, or requiring 60 ml. of 0.1 N alkali to neutralize 100 ml., is said to have an acidity of 60 degrees.

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[6] Shapiro *et al.: ibid.*, **2**, 121 (1944).

[7] Babkin: *Am. J. Digest. Diseases*, **5**, 107 (1937); **8**, 467 (1938).

[8] Thomas: *J. Am. Med. Assoc.*, **120**, 735 (1942).

[9] Ternberg and Eakin: *J. Am. Chem. Soc.*, **71**, 3858 (1949).

The hydrochloric acid of the gastric juice forms a medium in which the pepsin can most satisfactorily digest the protein food, and at the same time it acts to some extent as an antiseptic or germicide which prevents putrefactive processes in the stomach. When the hydrochloric acid of the gastric juice is diminished in quantity (hypoacidity) or absent, as it may be in many cases of functional or organic disease, there is no check to the growth of microorganisms in the stomach. There are, however, certain of the more resistant spores which even the normal acidity of the gastric juice will not destroy. A condition of hypoacidity may also give rise to fermentation with the formation of comparatively large amounts of such substances as lactic acid and butyric acid.

When free hydrochloric acid comes in contact with protein, as in the food, a reaction occurs with the formation of protein hydrochloride. This was formerly called "combined hydrochloric acid," but this term is so indefinite in its connotation that it should be abandoned (see p. 380). The formation of protein hydrochloride considerably raises the pH of gastric contents, since protein hydrochloride is a much less highly ionized acid than is hydrochloric acid itself. The reduction in hydrogen-ion concentration resulting from the formation of protein hydrochloride may permit processes which are acid-sensitive to proceed during gastric digestion, such as bacterial action or the action of salivary amylase (see p. 353).

Origin of Gastric Acid. The mechanism whereby the stomach produces a secretion which is about three million times more acid than the blood is not known. Many attempts have been made to solve this problem, and various theories have been proposed, none of which has received universal acceptance.

The currently favored view for the formation of acid by the parietal cells is based upon the discovery by Davenport[10] that the enzyme carbonic anhydrase (see Chapter 24) is present in large amounts in the parietal cells, and is relatively absent from the other cells of the gastric mucosa. This enzyme also occurs in red blood cells. It has been crystallized and found to contain 0.2 per cent zinc.[11] Potent inhibitors of carbonic anhydrase (like certain sulfa drugs) also depress hydrochloric acid secretion in the living animal.[12] Carbonic anhydrase catalyzes the hydration of carbon dioxide to carbonic acid, which dissociates in solution to yield hydrogen ions and bicarbonate ions:

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^+ + HCO_3^-$$

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[10] See Davenport: *Gastroenterology*, 1, 383 (1943); Gray: *ibid.*, 1, 390 (1943).

[11] Sumner and Somers: *Chemistry and Methods of Enzymes*, 3rd ed. New York, Academic Press Inc., 1953.

[12] Janowitz, Colcher, and Hollander: *Trans. N.Y. Acad. Sci.*, 15, 54 (1952).

If this reaction is pictured as occurring within the parietal cell, the carbon dioxide coming from metabolic processes, the hydrogen ions may be visualized as being secreted, along with an equivalent number of chloride ions, into the stomach while the bicarbonate ions enter the blood.

Here bicarbonate ion replaces chloride ion, which diffuses into the parietal cell and is available for excretion along with hydrogen ions into the gastric juice. Thus according to this theory the hydrogen ions of the gastric juice come from carbonic acid, and the chloride ions are derived from the blood. The replacement of blood chloride by bicarbonate should raise the pH of the blood, and in fact it has been shown that blood leaving the stomach during active gastric secretion is significantly more alkaline than the entering blood thus accounting for the "alkaline tide" (see p. 784).

It is an interesting consequence of this theory that the extra bicarbonate of gastric venous plasma as compared to gastric arterial plasma is the result of the direct entrance of bicarbonate as such into the plasma, and not the result of the entrance of anhydrous carbon dioxide into the blood followed by its hydration to carbonic acid in the red cell and diffusion from the red cell into the plasma; processes which are known to account for the extra bicarbonate of venous plasma over arterial plasma in other parts of the body. Now in ordinary venous plasma the increase in plasma bicarbonate due to diffusion of bicarbonate from the red cell into the plasma is associated with a chloride shift (see Chapter 24), chloride ions leaving the plasma and entering the red cell. In gastric venous plasma the increased plasma bicarbonate brings about a reversed chloride shift by diffusion into the red cell at the expense of chloride ions which diffuse out into the plasma. This has been cited as further evidence concerning the role of carbonic anhydrase in the formation of gastric acid. It should be remembered, however, that though this theory accounts for the production of acid by the parietal cell, no direct evidence concerning the actual mechanisms of formation and secretion is as yet available, and other theories[13] are not as yet untenable.

Pepsin. The most characteristic of the enzymes of the gastric juice is the proteolytic enzyme pepsin. Pepsin is a representative of a large group of enzymes, many of which are found in the gastrointestinal tract and all of which catalyze the hydrolytic splitting of the peptide bond, —CO—NH— to produce a free amino and a free carboxyl group. Within this group of peptide-splitting enzymes, two general types may be distinguished (Bergmann): (1) the proteinases or endopeptidases, and (2) the peptidases or exopeptidases. The endopeptidases act upon peptide linkages in both the central portion and the terminal portion of a polypeptide chain; the exopeptidases split peptide linkages in the terminal portion of the chain only. Differences between the various endo- and exopeptidases of the gastrointestinal tract are attributed largely to differences in the type and location of the amino acids united in the peptide bond, as will be evident in this and subsequent chapters. According to this classification, pepsin is an endopeptidase, since it can act upon peptide linkages within the large protein or polypeptide molecule as well as upon synthetic peptides (see below).

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[13] See Hollander: *Gastroenterology*, **1**, 401 (1943); *Federation Proc.*, **11**, 706 (1952).

Pepsin is apparently formed by the action of the hydrogen ions of the gastric juice on a precursor or zymogen, called *pepsinogen*, originating in the chief cells of the gastric mucosa. Both pepsin and pepsinogen are proteins and have been prepared in crystalline form;[14] crystalline pepsin appears, however, to be a mixture of enzymes rather than a single substance. Pepsinogen is more resistant to alkali than is pepsin. It does not clot milk at pH 5 nor liquefy gelatin at pH 4.7, but pepsin is active under these conditions. The formation of pepsin at pH 4.6 appears to be an autocatalytic reaction; i.e., the pepsin as it is formed acts upon further pepsinogen to yield still more pepsin. Since pepsin is known to act only on peptide linkages with a specific amino acid configuration (see below), it would appear that the activation of pepsinogen may involve the breaking of such linkages. The isoelectric point of pepsin is at such a low pH that it has not been accurately established.

Pepsin contains phosphorus. It is denatured and coagulated by heat, with the loss of peptic activity running parallel with the degree of denaturation. It is also inactivated and denatured in alkaline solutions (pH 10). In such cases there is some return of activity on acidification and standing; thus there has been a reversal of the denaturation of the pepsin protein. Pepsin is digested by trypsin in solutions more alkaline than pH 5.5. In more acid solutions trypsin is digested by pepsin. It has been shown that when pepsin is mixed with egg albumin in the pH range 3.1 to 4.2 a precipitate is formed which is not denatured protein.[15]

Pepsin acts very well at 40° C. The optimum pH is about 2.0, but this has been shown to be more related to the ionization of the substrate protein than to any effect on the enzyme itself. A variety of mineral and organic acids may be used to attain this pH with practically identical effects on the enzyme activity. At pH 4 its activity is very slight and at pH 5 it is stable but inactive. At pH values of 6.0 or greater it is unstable, and above pH 8 it is rapidly destroyed by OH ions.

Products of Peptic Hydrolysis. The gastric acid acting on food proteins at body temperature produces denatured proteins ("acid metaprotein"). Pepsin acts upon such denatured protein as well as upon native protein with the formation largely of protein derivatives of relatively low molecular weight (not over 1,000, according to Tiselius and Eriksson-Quensel[16]); such split products belong to the ill-defined class of the proteoses and peptones. In the normal time of gastric digestion, substances of this nature appear to be the chief end products of peptic action. Free tyrosine is also a frequent product of peptic digestion. The nature of the action of pepsin has been considerably clarified by the work of Bergmann, Fruton, and associates. They have studied the action of crystalline pepsin on synthetic substrate peptides of known chemical constitution. All synthetic substrates which were hydrolyzable by pepsin contained either tyrosine or phenylalanine in the molecule, with the peptide linkage specifically involving the *amino* group of these amino acids. If the tyrosine or phenylalanine was at the end of the peptide chain, hydrolysis proceeded more rapidly and the free amino acid was liberated, but peptic action was not limited to the presence of the amino acid at the end of a chain. Thus one may conclude that pepsin acts on peptide linkages associated with the presence of tyrosine or phenylalanine (although the possibility of other amino acids being concerned in peptic action is not necessarily excluded); the end product of the action will depend upon the relative position of these amino acids in the long chain of amino acid residues in the protein substrate. Pepsin acts upon practically all native proteins with the exception of keratin and the protamines; its lack of action in the case of keratins is believed to be due to the close packing of the polypeptide chains in the keratin molecule; in the case of protamines, to their deficiency in tyrosine and phenylalanine.

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[14] For discussion of crystalline pepsin see pp. 305 and 330. For preparation of crystalline pepsinogen see Herriott and Northrop: *Science*, **83**, 469 (1936); and Herriott: *J. Gen. Physiol.*, **21**, 501 (1938).

[15] Yasnoff and Bull: *J. Biol. Chem.*, **200**, 619 (1953).

[16] *Biochem. J.*, **33**, 1752 (1939).

In testing peptic activity at pH from 1 to 2.85 Currie and Bull[17] report that pepsin acts as a proteinase at the lower pH and splits fewer peptide linkages per mole of substrate thus yielding fragments of relatively high molecular weight. On the other hand, at a higher pH it acts as a peptidase and yields fragments of smaller molecular weight. As to the influence of temperature it was found that at 30° C. or below the digestion products possessed relatively low molecular weights whereas at temperatures between 35° C. and 50° C. the products of hydrolysis were of high molecular weight. The conclusion can therefore be drawn from these experiments that pepsin acts very efficiently at pH 2.85 and at about 30° C.

Rennin. Rennin is what is known as a milk-curdling or protein-coagulating enzyme. It is a proteinase which, acting upon the casein of milk, is believed to form first a soluble paracasein and a peptone-like body. In the presence of ionized calcium salts there is then formed an insoluble calcium paracaseinate which separates out as a curd. Rennin is commonly obtained from the mucosa of the fourth stomach of the calf and is used to curdle milk in cheese-making.

The matter was the subject of a long controversy, but it appears now to be clear that the enzyme, rennin, is an entity quite distinct from pepsin. It has very high milk-curdling power but practically no protein-digesting activity. In certain animals the curdling of milk is caused by pepsin. Pepsin can coagulate milk in practically neutral solution as contrasted with the high acidity required for its action on proteins in general.

Rennin acts best at a pH of 6.0 to 6.5 and at a temperature of about 45° C. Tauber and Kleiner have obtained a preparation curdling 4,550,000 times its weight of milk at pH 6.2 in 10 minutes at 40° C. The preparation has an isoelectric point of 5.4. It is apparently a diffusible proteose containing sulfur and is not coagulated by heat. It does not give the Millon and Hopkins-Cole tests. Rennin appears to exist in the calf's mucosa as prorennin, which is activated by the gastric acidity.

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[17] Currie and Bull: *J. Biol. Chem.*, **193**, 29 (1951).

The Action of Gastric Juice on Milk. A comparison between the large tough curds formed in the stomach from cow's milk and the small soft curds formed from human milk is shown in Figures 86 to 89, below.[18]

Gastric Lipase. A third enzyme present in gastric juice is a fat-splitting enzyme. It possesses but slight activity when the gastric juice is of normal acidity, but evinces its action principally at such times as a gastric juice of low acidity is secreted either from physiological or pathological cause. It thus may be of importance in the young animal where gastric acidity is considerably lower than in the adult. The digestion of fat in the stomach is, however, at most of but slight importance as compared with the digestion of fat in the intestine through the action of the

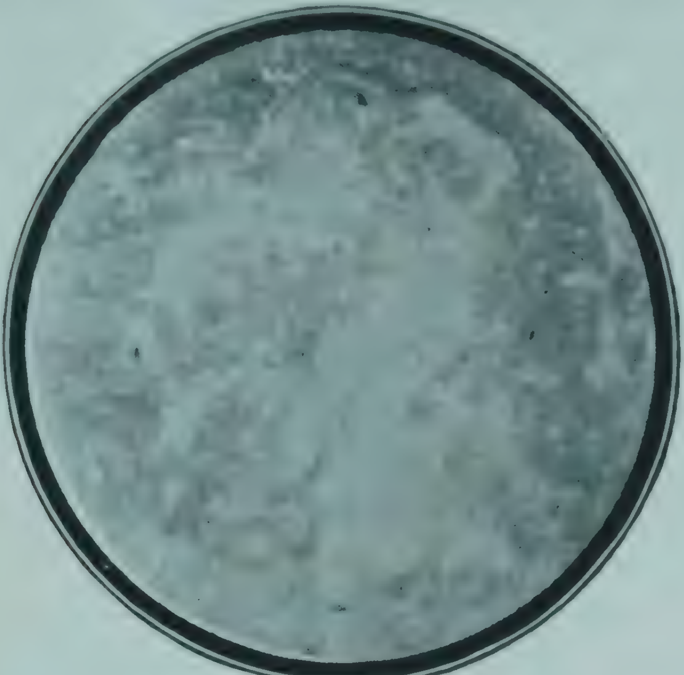

FIG. 86. CURD OF HUMAN MILK FIVE MINUTES AFTER INGESTION OF MILK.

Beginning of curd formation. One-half actual size.

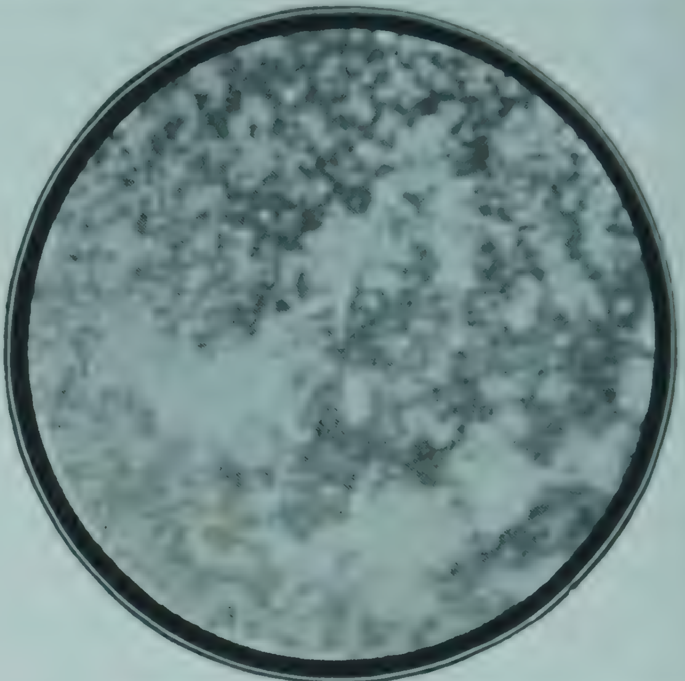

FIG. 87. CURD OF HUMAN MILK TEN MINUTES AFTER INGESTION OF MILK.

Maximum curd formation. One-half actual size.

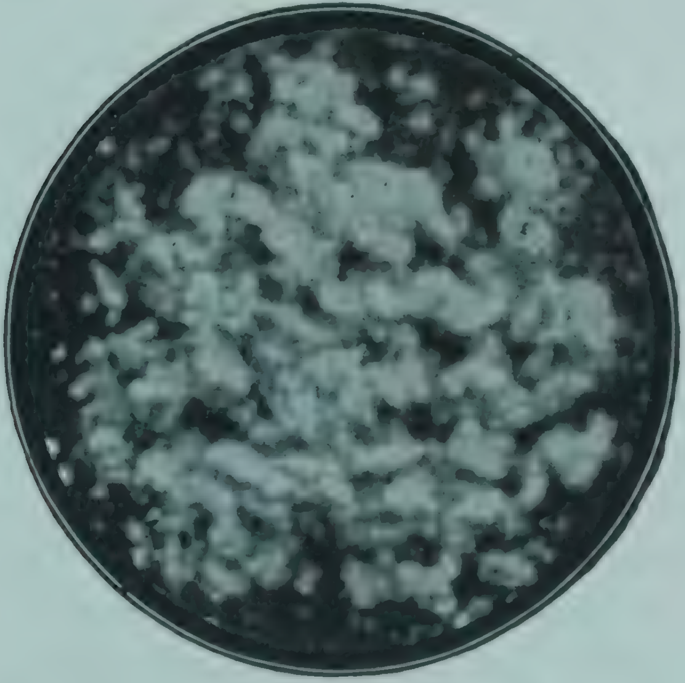

FIG. 88. CURD OF COW'S MILK REGURGITATED 10 MINUTES AFTER INGESTION OF 500 ML. OF WHOLE MILK.

One-half actual size.

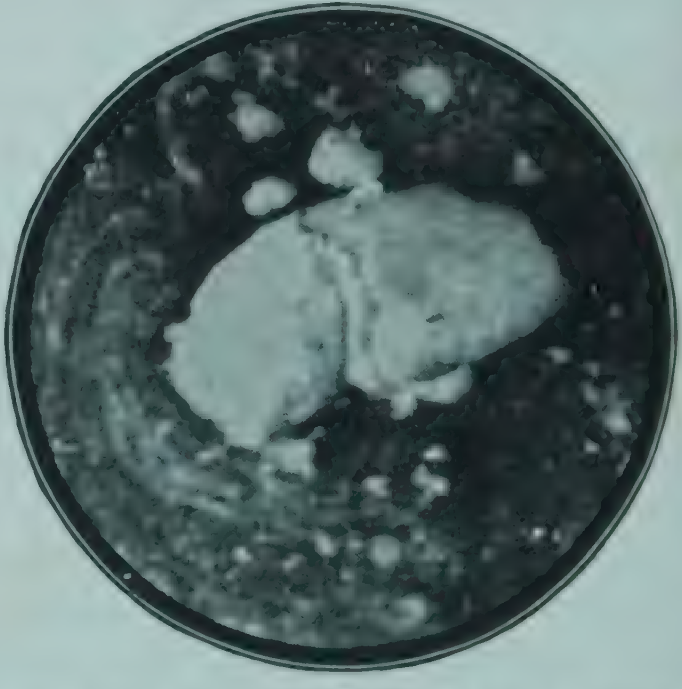

FIG. 89. CURD OF COW'S MILK REGURGITATED 25 MINUTES AFTER INGESTION OF 500 ML. OF WHOLE MILK.

One-half actual size.

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[18] Bergeim, Evvard, Rehfuss, and Hawk: *Am. J. Physiol.*, 48, 411 (1919).

lipase of the pancreatic juice (see p. 397). The presence of lipase in the gastric lumen, like that of trypsin, is probably due to regurgitation of intestinal contents through the pylorus.

NORMAL GASTRIC RESPONSE TO COMMON FOODS

On the basis of extensive studies made in the senior author's laboratory[19] the following table was constructed which contains data relative

EVACUATION TIMES AND HIGHEST TOTAL ACIDITIES FOR VARIOUS ARTICLES OF DIET

Articles of Diet (100-g. portions unless otherwise stated)	*Number of Observations*	*Highest Total Acidity (average) (ml. 0.1 N alkali to neutralize 100 ml. juice)*	*Evacuation Time (hours and minutes, average)*
Beef and beef products	25	120	3.00
Bread and cereals	75	80	2.40
Cakes	29	90	3.00
Chicken*	20	125	3.15
Egg and egg combinations	90	80	2.40
Fish*	75	130	2.50
Fruits*	68	90	2.00
Gelatin* (fruit-juice preparations)	5	70	2.00
Guinea hen*	2	110	4.00
Ice cream*	7	105	3.15
Ices*	4	65	2.35
Junket	4	65	2.25
Lamb and lamb products	14	135	3.00
Licorice	1	65	3.00
Milk:*			
Cow:			
400 ml	50	100	2.30
75 ml	3	45	1.15
Human:			
150 ml	5	60	1.40
225 ml	2	90	2.25
Nuts* (25 to 50 g.)	22	100	3.30
Orange-albumin (2:1)	2	85	2.20
Pies	29	90	2.30
Popcorn	3	60	1.30
Pork and pork products	31	120	3.15
Puddings	23	90	2.20
Sugars and candies	28	70	2.05
Turkey*	2	140	3.30
Veal:*			
(*a*) Market	7	140	2.50
(*b*) "Bob"	7	110	3.20
Vegetables prepared in different ways	124	75	2.15

* Unpublished data.

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[19] See *Researches and Writings*, Philip B. Hawk and collaborators, published and distributed privately, 1942.

to the evacuation time and the highest total acidity after the ingestion of certain common foods by normal men. In the tests here summarized 100-g. portions of food (unless otherwise stated) were fed to normal men and the gastric reponse determined by the fractional method (see p. 383). It will be noted in general that foods such as meats which are high in protein, and for which gastric digestion is hence of the greatest importance, remain longest in the stomach (3 to 4 hours) and give rise to the highest acidities (120 or higher). Foods low in protein, such as fruits and many vegetables, leave the stomach soon (1.5 to 2 hours) and give rise to much less secretion. Foods intermediate in protein content, such as cereal foods, show intermediate acidities and emptying times. Milk has considerable buffer action. Water leaves the stomach rapidly.

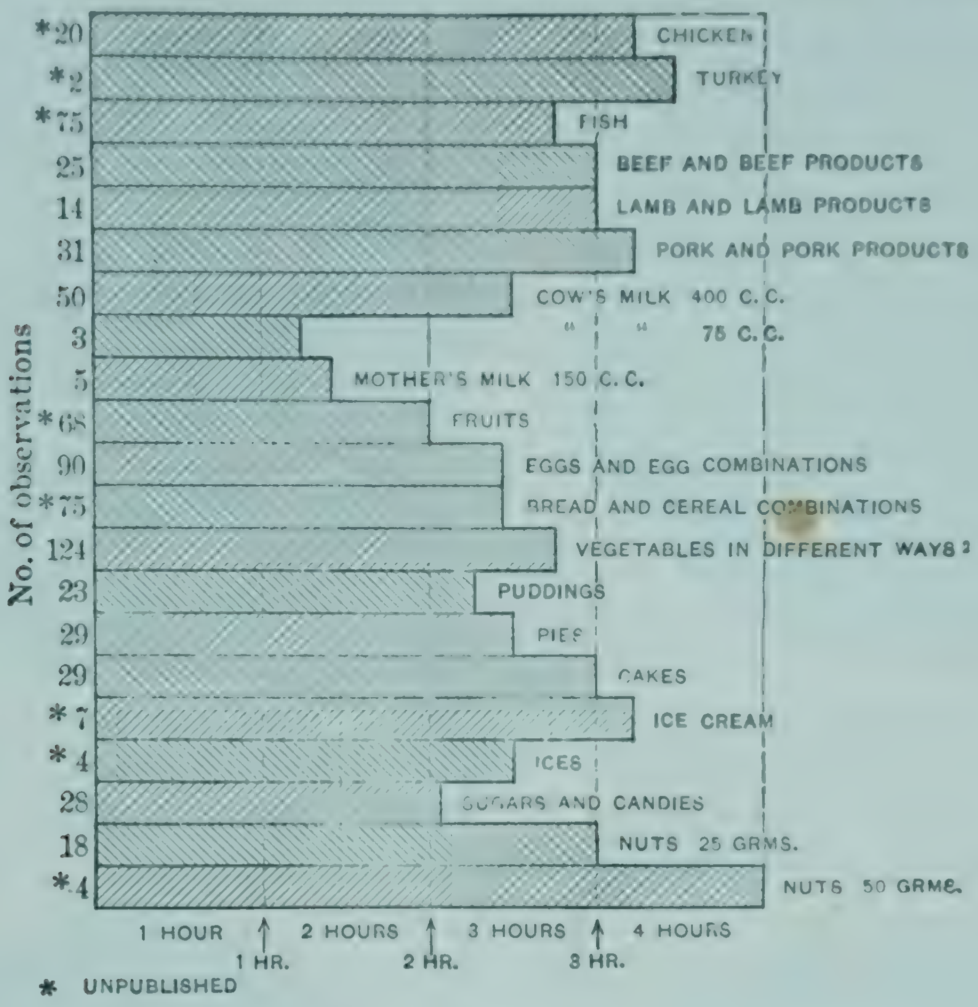

FIG. 90. EVACUATION TIMES OF COMMON FOODS.
Hawk, Rehfuss, and Bergeim: *Am. J. Med. Sci.*, **171**, 359 (1926).

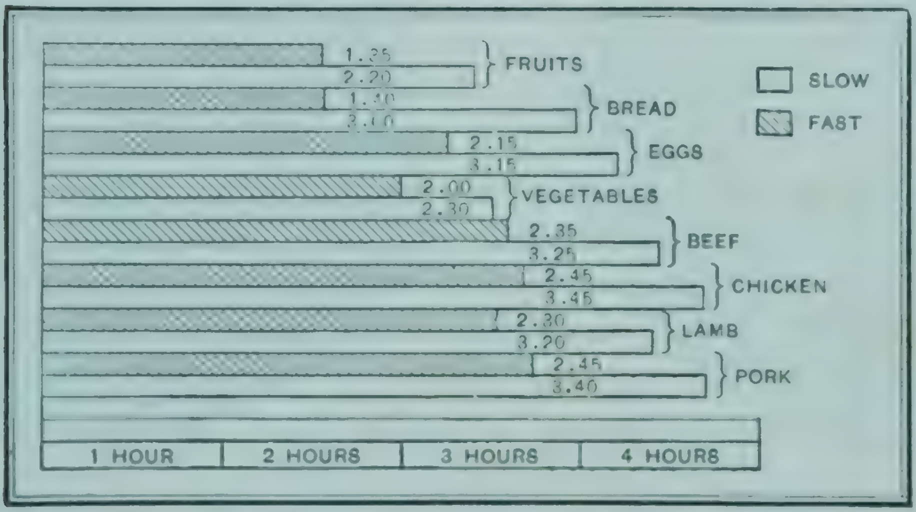

FIG. 91. THE EVACUATION TIMES OF FAST AND SLOW STOMACHS.
Hawk, Rehfuss, and Bergeim: *Am. J. Med. Sci.*, **171**, 359 (1926).

The relationship of the average evacuation times of the various foods is shown graphically in Fig. 90, whereas the variation in the evacuation time of the same food by fast and slow stomachs is shown in Fig. 91.

COLLECTION OF HUMAN GASTRIC JUICE

Have one or more volunteers from the class take the Rehfuss stomach tube as directed on p. 384. The subjects must omit breakfast if the tube is taken in the morning, or luncheon if taken in the afternoon. Empty the stomach (see pp. 384–385) and, with the tube still in place, allow each subject to drink 250 ml. of water. The water will stimulate the flow of gastric juice and will itself quickly leave the stomach. In many instances fairly concentrated gastric juice may be obtained from the stomach in from 30 to 45 minutes after the introduction of the water. Remove this gastric juice according to procedure outlined on p. 386. For the composition of human gastric juice see p. 377. See also Exp. 9, p. 373. If thought desirable, the gastric juice resulting from psychical stimulation (see p. 373) or following histamine injection (see p. 385) may be collected instead of that following the chemical stimulation of water. (Curves showing the stimulatory power of water are given in Fig. 92.)

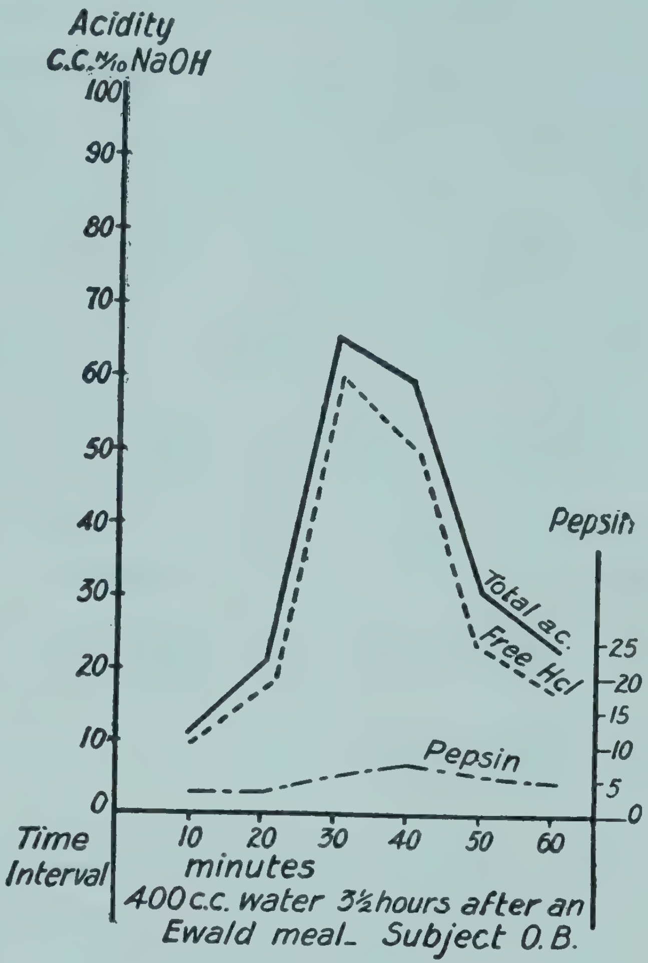

FIG. 92. CURVES SHOWING STIMULATORY POWER OF WATER.

Bergeim, Rehfuss, and Hawk: *J. Biol. Chem.*, **19**, 345 (1914).

PREPARATION OF ARTIFICIAL GASTRIC JUICE

1. *From Pig's Stomach.* Dissect the mucous membrane of a pig's stomach from the muscular portion and discard the latter. Divide the mucous membrane into two parts (four-fifths and one-fifth). Cut up the larger portion, place it in a large-sized beaker with at least 4 volumes of 0.4 per cent hydrochloric acid, and keep at 38° to 40° C. for at least 24 hours. Add more HCl as needed to keep the mixture acid to Congo red paper; otherwise putrefaction may occur. Filter off the residue, consisting of nuclein and other substances, and use the filtrate as an artificial gastric juice. This filtrate contains pepsin and the products of the digestion of the stomach tissues; i.e., denatured protein, proteoses, and peptones.

2. *From Commercial Pepsin.* Dissolve 750 mg. of U.S.P. pepsin in 100 ml. of 0.1 N hydrochloric acid.

PREPARATION OF A GLYCEROL EXTRACT OF PIG'S STOMACH

Take the one-fifth portion of the mucous membrane of the pig's stomach not used in the preparation of the artificial gastric juice, cut it up finely, place it in a small-sized beaker, and cover the membrane with glycerol. Stir frequently and allow to stand at room temperature for at least 24 hours. The glycerol will extract the pepsinogen. With a pipet or by other means, sepa-

rate the glycerol from the pieces of mucous membrane and use the glycerol extract as required in the later experiments.

PRODUCTS OF GASTRIC DIGESTION

Into the artificial gastric juice, prepared as above described, place the protein material (fibrin, coagulated egg white, or lean beef) provided for you by the instructor, add 0.4 per cent hydrochloric acid as suggested by the instructor, and keep the digestion mixture at 40° C. for two to three days. Stir frequently and keep free hydrochloric acid present in the solution as indicated by a blue color with Congo red paper.

The original protein has been digested and the solution now contains the products of peptic proteolysis; i.e., denatured protein, proteoses, peptones, etc. The insoluble residue may include nuclein and other substances. Filter the digestion mixture, and after testing for free hydrochloric acid neutralize the filtrate with sodium hydroxide solution. If any of the denatured protein is still untransformed into proteoses, it will precipitate upon neutralization. If any precipitate forms, heat the mixture to boiling and filter. If no precipitate forms, proceed without filtering.

We now have a solution containing a mixture consisting principally of proteoses and peptones. Separate and identify the proteoses and peptones according to the directions given on p. 197.

GENERAL EXPERIMENTS ON GASTRIC DIGESTION

1. *Conditions Essential for the Action of Pepsin.* Prepare four test tubes as follows:

(*a*) Five ml. of pepsin solution.
(*b*) Five ml. of 0.4 per cent hydrochloric acid.
(*c*) Five ml. of pepsin–hydrochloric acid solution.[20]
(*d*) Two or 3 ml. of pepsin solution and 2 to 3 ml. of 0.5 per cent sodium carbonate solution.

Into each tube introduce a small piece of fibrin and place tubes in the incubator or water bath at 40° C. for one-half hour, carefully noting any changes which occur.[21] (Carmine-fibrin[22] may be used to advantage in this and the following tests under Gastric Digestion. In this case, however, the experiments should be conducted at room temperature.) Now combine the contents of tubes (*a*) and (*b*) and see if any further change occurs after standing at 40° C. for 15 to 20 minutes. Explain the results obtained from these five experiments.

2. *Influence of Different Temperatures.* In each of five test tubes place 5 ml. of pepsin–hydrochloric acid solution. Immerse one tube in ice water, keep a second tube at room temperature, place a third in the incubator or water bath at 30° C., and keep the fourth at 50° C. Boil the contents of the fifth tube for a few moments; then cool and also keep it at 30° C. Into each tube intro-

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[20] 0.75 per cent commercial pepsin in 0.4 per cent HCl may be used.

[21] Digestion of fibrin in a pepsin-hydrochloric acid solution is indicated first by a *swelling* of the protein due to the action of the acid, and later by a *disintegration* and *solution* of the fibrin due to the action of the pepsin–hydrochloric acid. If uncertain at any time whether digestion has taken place, the solution under examination may be filtered and the biuret test applied to the filtrate. A positive reaction will signify the presence of proteoses (albumoses) or peptones, the presence of which would indicate that digestion has taken place. The biuret reaction must be more positive than that given by the pepsin–hydrochloric acid solution alone.

[22] See Appendix.

duce a small piece of fibrin and note the progress of digestion. In which of the tubes does the most rapid digestion occur? Explain this.

3. *The Most Favorable Acidity.* Prepare three tubes as follows:

(*a*) 3 ml. of pepsin solution + 3 ml. of 0.4 per cent HCl. (Acidity about 0.2 per cent HCl or pH 1.3.)

(*b*) 3 ml. of pepsin solution + 1 ml. of 0.4 per cent HCl + 2 ml. of water. (Acidity about 0.067 per cent HCl or pH 1.8.)

(*c*) 3 ml. of pepsin solution + 4 drops or 0.2 ml. of 0.4 per cent HCl + 3 ml. of water. (Acidity about 0.013 per cent HCl or pH 2.5.)

Introduce a small piece of fibrin into each tube, keep them at 40° C., and note the progress of digestion. In which acidity does pepsin act best on fibrin? The acid decreases during digestion due to its combination with protein, so that as a determination of optimum pH this procedure is not exact. The optimum for pepsin is about pH 1.8 under the conditions of this experiment.

4. *Differentiation Between Pepsin and Pepsinogen.* Prepare five tubes as follows:

(*a*) Few drops of glycerol extract of pepsinogen + 2 to 3 ml. of water.

(*b*) Few drops of glycerol extract of pepsinogen + 5 ml. of 0.2 per cent hydrochloric acid.

(*c*) Few drops of glycerol extract of pepsinogen + 5 ml. of 0.5 per cent sodium carbonate.

(*d*) Two or 3 ml. of pepsin solution + 2 to 3 ml. of 1 per cent sodium carbonate.

(*e*) Few drops of glycerol extract of pepsinogen + 5 ml. of 1 per cent sodium carbonate.

Add a small piece of fibrin to the contents of each tube, keep the five tubes at 40° C. for one-half hour, and observe any changes which may have occurred. To (*a*) add an equal volume of 0.4 per cent hydrochloric acid, neutralize (*c*), (*d*), and (*e*) with hydrochloric acid, and add an equal volume of 0.4 per cent hydrochloric acid. Place these tubes at 40° C. again and note any further changes which may occur. What contrast do you find in the results from the last three tubes? On the basis of these tests, what is the relative resistance of pepsin and pepsinogen to alkalies?

5. *Comparative Digestive Power of Pepsin with Different Acids.* Prepare a series of six test tubes each containing 5 ml. of a solution of one of the following acids[23] each solution having the same acidity of pH 2: (1) HCl, (2) H_2SO_4, (3) H_3PO_4, (4) oxalic acid, (5) lactic acid, (6) acetic acid. Add 2 ml. of 0.5 per cent pepsin solution or 1 ml. of the glycerol extract of the hog's stomach and a small piece of fibrin. Put in a water bath and keep at 40° C. Note the progress of digestion. Can you confirm the findings of other observers[24] that it is the hydrogen-ion concentration (pH), rather than the nature of the acid present, which is the controlling factor in the influence of acids on peptic digestion?

Titrate 10 ml. of each of these acids (lactic and acetic acids only 1 ml.)

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[23] HCl 0.037 per cent, H_2SO_4 0.059 per cent, H_3PO_4 0.2 per cent, oxalic acid crystals 0.15 per cent, lactic acid 7.2 per cent, and acetic acid 18 per cent. These solutions are best checked for pH by adding a few drops of thymol blue solution to 5 ml. of each in test tubes and if necessary adding more acid to get the same color as obtained with the oxalic acid, which can be weighed out accurately.

[24] Northrop (*J. Gen. Physiol.*, 1, 607 (1919); 5, 263 (1922)) finds, however, that acetic acid is slightly less effective than the others apparently because of some action of the acetic acid not on the enzyme but on the protein.

with 0.1 N NaOH, using phenolphthalein as an indicator. Calculate the normality of each. Six students may conveniently work together on this part of the experiment, each student then assembling the entire data. What does this experiment teach as to the relationship of hydrogen-ion concentration to titratable acidity and as to the biological significance of each?

6. *Quantitative Determination of Peptic Activity.* See Chapter 15, Gastric Analysis.

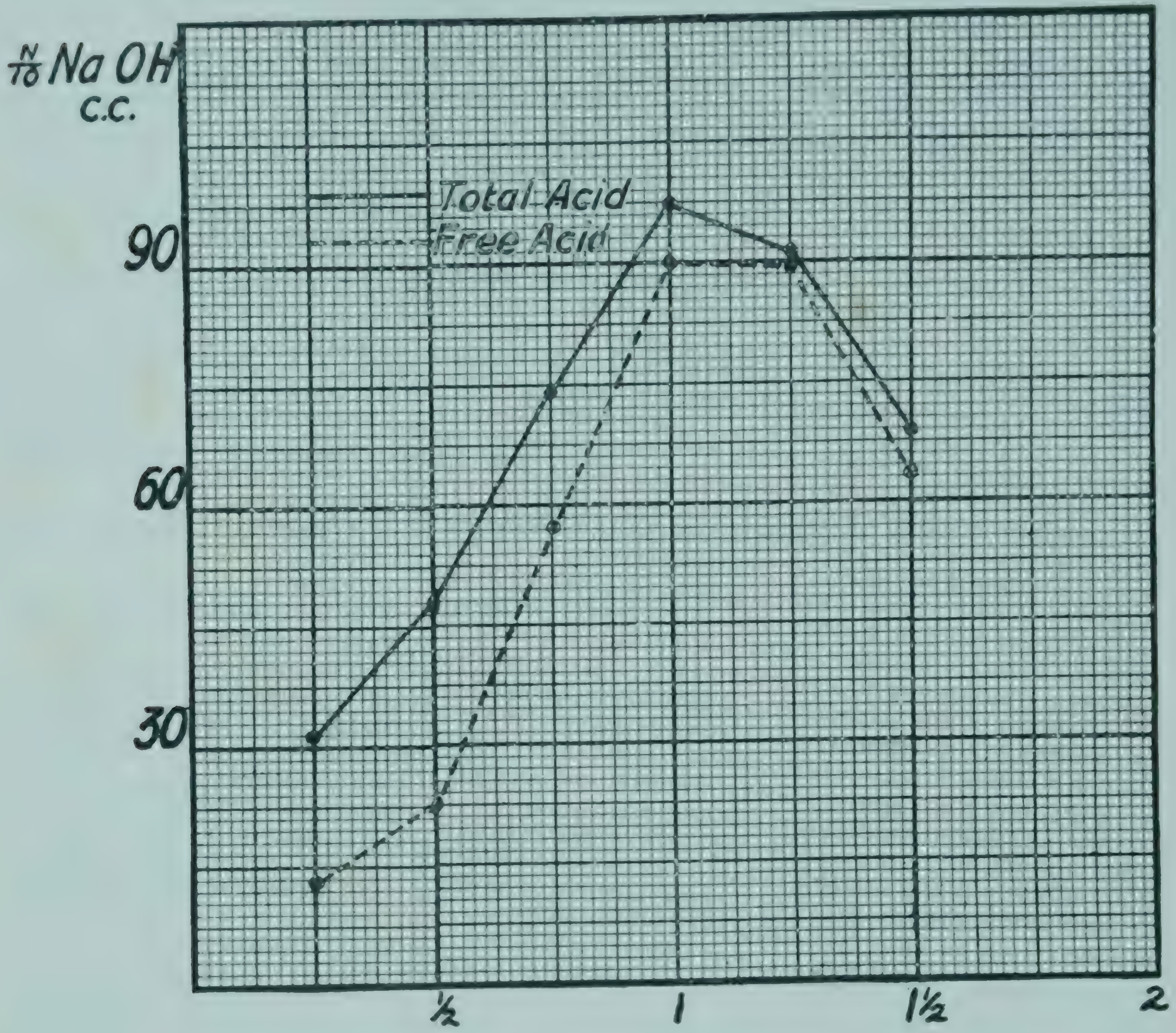

FIG. 93. CURVES SHOWING STIMULATORY POWER OF BEEF EXTRACT.
From unpublished data collected in the senior author's laboratory by Dr. Chester C. Fowler.

7. *Quantitative Determination of Rennin.* Prepare a standard milk of pH 5.0 by mixing equal volumes of fresh milk and M acetate buffer of pH 5.0. Introduce 10-ml. portions of this milk into a series of test tubes and keep at 20° C. Add 1-ml. portions of various dilutions of a rennin solution.[25] Mix and note the time of clotting. The amount of rennin that clots 1 ml. of the buffered milk in 10 minutes at 20° C. is called one unit of rennin.

8. *Characteristics of Human Gastric Juice.* Take some of the human gastric juice collected as described on p. 369 and show that it is acid in reaction, that it contains chlorides, and that it has the power to digest protein material and to curdle milk.

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[25] Any good commercial rennet may be used in preparing this solution.

9. ***Chemical and Psychical Stimulation of Gastric Secretion.*** **Have one or more volunteers from the class swallow the Rehfuss stomach tube as directed on p. 384. The subjects must omit breakfast if the tube is taken in the morning or luncheon if taken in the afternoon. Empty the stomach (see pp. 384–385) and, with the tube still in position, allow each subject to drink 250 ml. of bouillon prepared by dissolving one bouillon cube in hot water. Collect samples of gastric contents at intervals until the stomach is empty as described under Section 5 on p. 386. The samples thus collected may be examined qualitatively for acid, chlorides, pepsin, and rennin; or they may be submitted to the quantitative procedure given on p. 386. If the examination is made quantitative the data may be recorded in the form of a curve such as shown in Fig. 93.**

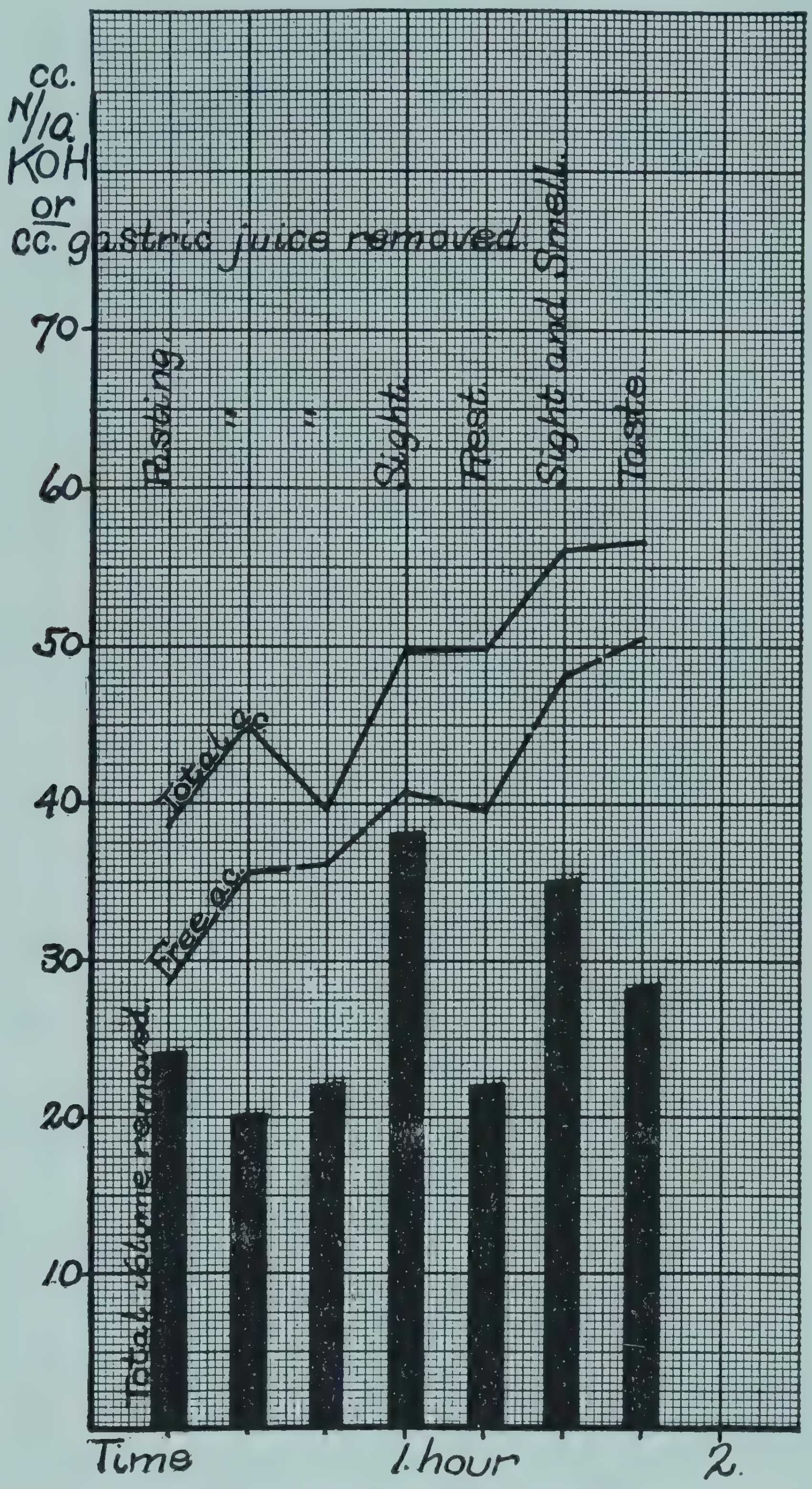

FIG. 94. CURVES SHOWING PSYCHICAL STIMULATION OF GASTRIC SECRETION.

Miller, Bergeim, Rehfuss, and Hawk: *Am. J. Physiol.*, **52,** 1 (1920).

For the ***psychical*** **stimulation, empty the stomach as above. Then instead of having the subjects drink bouillon, simply permit them to** ***see*** **and** ***smell***

an appetizing beefsteak while it is being cooked. Collect samples of gastric contents as above, plot a curve and compare with Fig. 94.

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15

Gastric Analysis

The method of gastric analysis which was in vogue clinically for years entailed the feeding of a standard test meal, the removal of the complete stomach contents at the end of a one-hour period, and the analysis of the material so removed. That this method is inaccurate has been repeatedly demonstrated in the senior author's laboratory and elsewhere. Furthermore, owing to the bulk of the old form of stomach tube and the discomfort occasioned by its use, it is impossible to follow the whole cycle of digestion and estimate, step by step, the exact changes which take place in the stomach after the introduction of definite food mixtures into that organ.

Realizing the inadequacy of the procedure entailed in the old method of gastric analysis, an improved procedure was developed by Dr. Martin E. Rehfuss in the senior author's laboratory. This so-called Fractional Method entails the analysis of samples of material withdrawn from the stomach (by syringe) at short intervals for a period of two hours or more (until the stomach is empty) after the ingestion of the test meal. By this means the observer is able to follow the entire cycle of gastric digestion and is not limited, as in the old method, to information derived from the analysis of a single sample of stomach contents withdrawn at the end of one hour. That the acid values obtained by the old method may be grossly misinterpreted and lead to an incorrect diagnosis is indicated by the diagram shown in Fig. 95.

It is set forth in Fig. 95 that various types of abnormal gastric secretion would be considered normal on the basis of a single examination at the end of one hour, whereas the application of the fractional method reveals an abnormality of the secretion. The removal of samples of gastric contents at short intervals, for a period of two hours or more after a test meal, is made possible by the use of a modified stomach tube[1] of small diameter (No. 12 French tubing) and fitted with a metal tip. The tip is slotted with large perforations, the diameter of each being equivalent to the maximum bore of the tubing. Such a tube can be left in the stomach through the entire cycle of gastric digestion without inconvenience to the patient. The Rehfuss stomach tube is shown in Fig. 96.[2] Lyon suggested a modified tip.

A tube much favored in England is that devised by Ryle.[3] This consists of a small-bore rubber tube with a blind end, into which is inserted an oval weight of lead. Holes are punched in the rubber tube just above the weight.

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[1] Rehfuss: *Am. J. Med. Sci.*, **147**, 848 (1914).

[2] This tube is manufactured by Charles Lentz and Sons, Philadelphia.

[3] Ryle: *Gastric Function in Health and Disease*, London, Oxford University Press, 1926.

Other tubes or plastic catheters have been employed in gastric analysis.[4] Certain of these (Levin) may be passed through the nose. Such tubes are useful in rapidly demonstrating the absence or presence of free hydrochloric acid in the stomach contents.

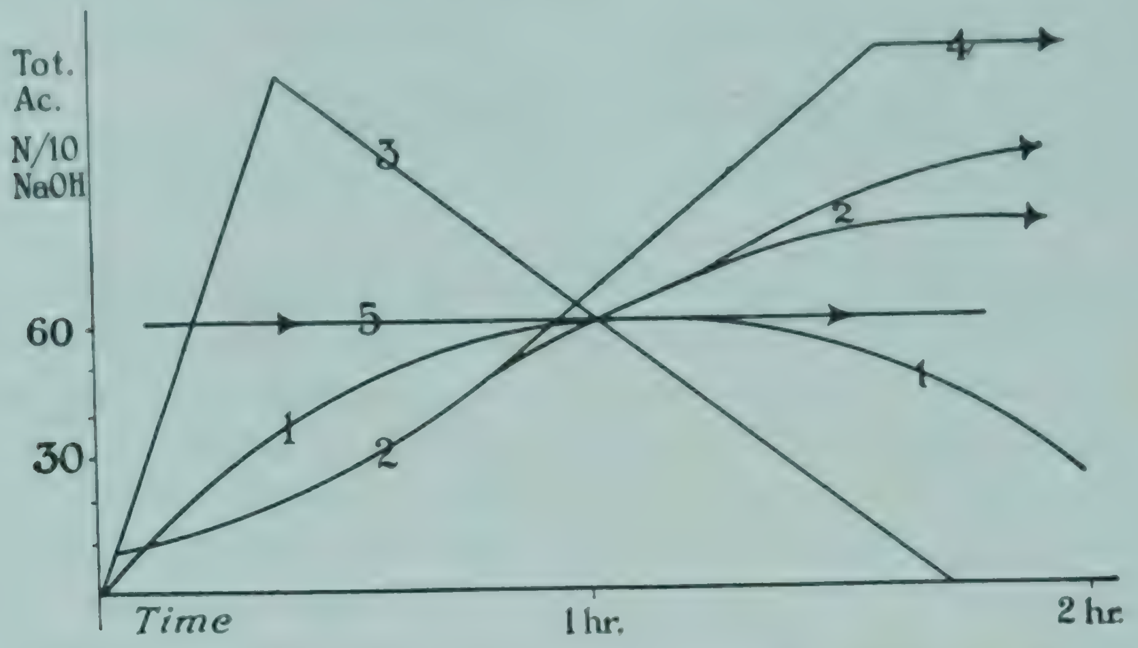

FIG. 95. NORMAL AND PATHOLOGICAL CURVES AFTER AN EWALD MEAL.

(1) Normal curve, (2) delayed digestion with late hyperacidity, (3) larval hyperacidity, (4) tardive hyperacidity, and (5) marked continued secretion from obstruction.

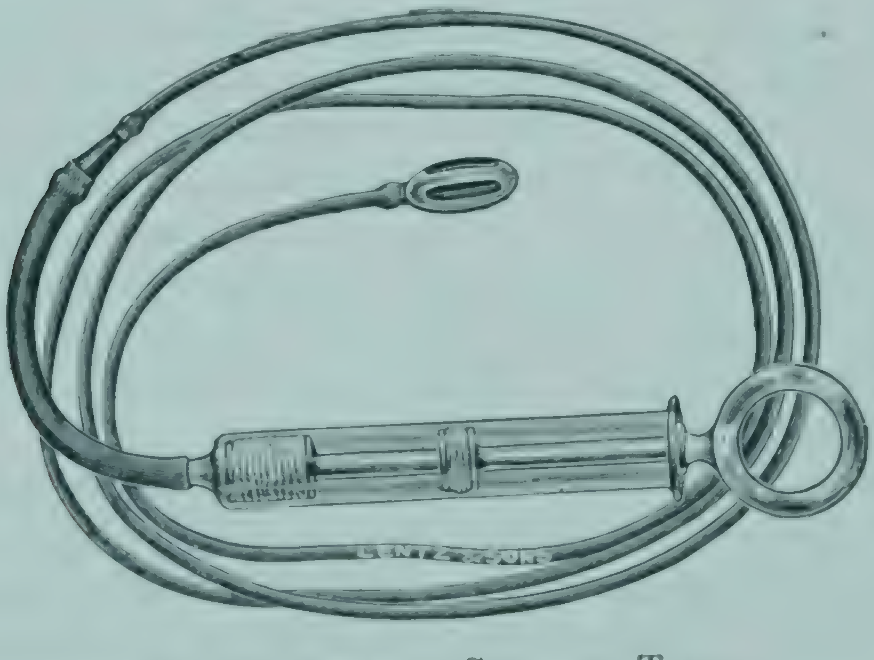

FIG. 96. REHFUSS STOMACH TUBE.

A method of determining gastric acidity *without intubation* has also been suggested by Segal and his associates.[5] An editorial in the Journal of the American Medical Association[6] discusses the method as follows:

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[4] One of these, the *Sawyer tube*, was developed at the Mayo Clinic by Miss Catherine Sawyer.

[5] Segal, Miller, and Morton: *Proc. Soc. Exptl. Biol. Med.*, **74**, 218 (1950); Segal, Miller, Morton, and Young: *Gastroenterology*, **16**, 380 (1950); Segal: *Med. Clin. N. Amer.*, 35, 593 (1951).

[6] *J. Am. Med. Assoc.*, **146**, 260 (1951).

"Segal and his associates recently described a test for determining the presence or absence of free hydrochloric acid in the stomach without subjecting the patient to intubation. The principle of the test lies in the use of a cation exchange resin to which is attached a special indicator cation that can be readily identified when it is released from the compound by an ion exchange reaction. Clinical trials have been based on administration of a quininium exchange indicator compound[7] prepared by subjecting an acid-conditioned cation exchange resin to a solution of quinine hydrochloride. By this treatment, quinine replaces the hydrogen cations of the carboxylic acid groups present in the resin. If free hydrochloric acid is present in the stomach, the quinine will be replaced by hydrogen ions and the liberated quinine will be absorbed into the body and can later be detected in the urine. Smaller amounts of quininium cations may be displaced by the cations present in the secretions of the small intestine. However, these quininium cations can be differentiated from those displaced by the hydrogen ions in gastric juice by the time of appearance and the amount of quininium cations in the urine.

This procedure is more pleasant for the patient than the conventional intubation method. Its simplicity suggests its usefulness as a screening test to select achlorhydric subjects in the gastric cancer age group for further investigative studies."

PROPERTIES AND COMPOSITION OF HUMAN GASTRIC JUICE

	Appetite Juice	*Residuum*
Specific gravity	1.007	1.006
Freezing point depression, °C	−0.55°	−0.47°
Total acidity, per cent HCl	0.45	0.30
Total solids, g. per 100 ml	0.55	0.98
Organic solids, g. per 100 ml	0.41	0.53
Inorganic solids, g. per 100 ml	0.14	0.45
Total nitrogen, g. per 100 ml	0.060	0.066
Total phosphorus, g. per 100 ml	..	0.005
Total sulfur, g. per 100 ml	..	0.007
Ammonia N, g. per 100 ml	0.002–3	..
Amino-acid N, g. per 100 ml	0.003–9	..
Chlorides (as Cl), g. per 100 ml	0.5	..

An apparatus has also been devised[8] for the determination of intragastric conductance and temperature. The apparatus is also provided with an aspiration tube similar to that of the Rehfuss tube which makes possible the removal of samples of gastric contents for chemical analysis.

For a long time the consensus, based principally upon the work of the Pavlov school, was to the effect that the gastric juice of normal man had an average acid concentration of 0.2 per cent hydrochloric acid, whereas the gastric juice of the dog and cat had an average acid concentration of 0.4 to 0.5 per cent hydrochloric acid. These experiments were based principally upon the examination of the pure gastric juice of the lower animals as compared with the stomach contents of man. Later experiments, however, demonstrated that the acid concentration of the *freshly*

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[7] Diagnex (Squibb) is useful in this connection. The appearance of quinine in the urine within two hours after the administration of Diagnex shows the presence of HCl. The absence of quinine within this period indicates achlorhydria.

[8] Bergeim: *Am. J. Physiol.*, **45**, 1 (1917).

secreted gastric juice of man is similar to that of the dog; i.e., 0.4 to 0.5 per cent hydrochloric acid. Boldyreff claimed that this initial high acidity of the human gastric juice is normally lowered to the "optimum acidity" of 0.15 to 0.2 per cent by regurgitation of alkaline fluids (bile, pancreatic, and intestinal juices) from the intestine. This constitutes what Boldyreff termed "the automatic regulation of gastric acidity." This claim has been amply substantiated. Both bile and trypsin are easily identified in the stomach contents of man after the introduction of 0.5 per cent hydrochloric acid into the empty organ.

The composition of human gastric juice and of the residuum (see p. 384) is given in the table on p. 377.

THE USE OF INDICATORS IN GASTRIC ANALYSIS

Indicators are used in gastric analysis for two main purposes: (1) For the colorimetric determination of the pH of gastric contents, and (2) as an aid in the measurement of gastric acidity by titration. The use of indicators for the first purpose is identical in principle with their use in this connection with other fluids, and is described in detail on p. 37. The practical details in connection with gastric analysis are presented on p. 379.

The primary objective in the titration of gastric acidity is to determine the amount of unneutralized hydrochloric acid present ("free hydrochloric acid"), in the possible presence of other acids which while titratable are nevertheless so much less highly ionized than hydrochloric acid that they contribute little or nothing to the hydrogen-ion concentration of the solution. It is the hydrogen-ion concentration (i.e., the pH) of the gastric contents which to a large extent determines whether or not the physiological functions of the gastric secretion will be served, and hydrogen ions in concentration sufficient to maintain a normal pH can come only from a highly ionized acid such as hydrochloric acid. Thus gastric function can be evaluated in terms of the presence and amount of free hydrochloric acid. Since the concentration of free hydrochloric acid determines the pH, it is clear that a pH measurement will frequently give as much information as a titration, and increasing use of pH determination in this connection is being made clinically, particularly in view of the ease with which precise pH determinations may be made with the modern electronic pH meters (see Chapter 1).

The establishment by titration of the presence and amount of free hydrochloric acid in the presence of other titratable acids is based on the fact that hydrochloric acid is completely dissociated in solution, the hydrogen ions from this dissociation reacting with the added OH ions before any undissociated acid present can ionize and so react. Thus the amount of alkali added up to the point of practically complete neutralization of the hydrochloric acid present should be distinguishable from that necessary for the remaining acid or acids. That this is so is evident from an inspection of the titration curve for hydrochloric acid as compared with that for a typical weak acid such as acetic acid; such curves are shown in Fig. 11 of Chapter 1. From an inspection of these curves it can be seen that as standard alkali is added to a solution containing hydrochloric acid, the pH of the solution changes relatively little until most of the acid is neutralized, increasing from pH 1 to about pH 2, when about three-fourths of the acid has been neutralized. As the titration continues, at about pH 3.5 or so it is clear that practically all of the acid has been titrated; stopping the titration at this point gives a value for the acid present which is almost indistinguishable from the value obtained if the titration were carried to the end point with such indicators as methyl orange, litmus, or phenolphthalein.

The situation is quite different if a weak acid, such as acetic acid for example, is being titrated. In the case of acetic acid, the solution has a pH of about 3 before any alkali has been added, this pH corresponding to the relatively small (about 1 per cent) ionization of the acetic acid molecules. As the pH is increased by the addition of alkali, more of the acetic acid dissociates to give hydrogen ions which are capable of reacting with the added OH ions, until ultimately sufficient alkali has been added to neutralize all of the acid initially present. But it will be noted that the end point of this titration (i.e., the pH at which equivalent amounts of alkali and acid are present) is not pH 7 but rather nearer pH 8.5. Thus, in order to titrate such an acid as this, it is necessary to use an indicator which changes color at about pH 8.5; phenolphthalein is such an indicator.

From what has just been said, it follows that if a mixture of hydrochloric acid and some weak acid or acids is being titrated with standard alkali, the buret reading at pH 3.5 or thereabouts will be a measure of the hydrochloric acid present, while the reading at pH 8.5 will be a measure of the total acidity of the solution. It is thus possible to distinguish quantitatively between these two types of acidity provided that means of indicating the pH of the solution are available. This may be done using a pH meter; it is much more common to select an indicator whose color change lies at the pH range desired. Of the various indicators which have been proposed for this purpose in gastric analysis, *Töpfer's reagent* (dimethylaminoazobenzene) and phenolphthalein are almost universally used. Töpfer's reagent has a color change from red to yellow over the pH range 2.9 to 4.0 (see Table), the intermediate color of salmon pink being noticeable at approximately pH 3.3. Thus if gastric contents are titrated with alkali to the color change with Töpfer's reagent, a measure of the free hydrochloric acid present will be obtained, the value being uninfluenced by any weak acids which may be present. If the titration is then continued to the color change with phenolphthalein (pH 8.5), the total acidity is determinable.

Töpfer's reagent has a number of disadvantages; the color change is not sharp and requires a certain amount of familiarity before the proper end point is routinely obtainable; furthermore, the color fades rapidly at the end point and thus precludes the setting up of pH controls for the more precise establishment of the end point. Other indicators have been proposed, such as thymol blue (red, becoming yellow at pH 2.8), and bromophenol blue (yellow, beginning to turn blue at pH 3.4 or so), but in the authors' experience of teaching medical students and technicians the routine of gastric titrations, neither of these has proved so satisfactory as Töpfer's reagent.

In the above discussion acetic acid was used as an example of a typical weak acid, to illustrate the principles involved in a gastric titration. In actual practice the weak acids which may be found in gastric contents include *protein hydrochloride* (so-called "combined hydrochloric acid"), *acid phosphates*, and various organic acids such as *lactic*, *citric*, etc., after fermentation or the ingestion of certain foods. At one time the mistaken notion prevailed that by the suitable use of various indicators it was possible to differentiate between these components of the weak-acid fraction of gastric contents. This is not true, since the titration curves of these various components overlap to such an extent that it is impossible to differentiate between the contribution of each to the total acidity, and the concept should be abandoned. Even the distinction between free mineral acid and weak organic acids becomes less sharp if the organic acids have an appreciable ionization at pH 3 or so, as is the case for example with lactic acid. The presence of significant amounts of such organic acid in gastric contents is quite unusual; should it occur, the determination of volatile chloride is of value in establishing the extent of acidity due to hydrochloric acid.

It is occasional practice in the titration of relatively pure gastric contents to subtract the value for the free acidity from that of the total acidity and call the difference the "combined acid." The validity of this is questionable; even assuming that the difference between free and total acid is partly due to acid which has reacted with protein to form protein hydrochloride, it is clear that the amount of acid which has so reacted is measurable only by titrating the solution to the isoelectric point of the protein; further titration beyond this pH represents the formation of alkali salt of protein and will depend on the amount and nature of protein present, without reference to how much hydrochloric acid has been "combined" with the protein. Actually, the difference between free and total acid is more a measure of the buffer power of the gastric juice than anything else. From a practical point of view, therefore, it would appear that the requirements of gastric analysis, at least in so far as gastric acid is concerned, are largely met by measurement of either the free acid or the pH.

The table on p. 381 lists the characteristics of those indicators which have found application in gastric analysis and for other purposes. Experiments which follow illustrate the application of the principles just presented.

Tests with Indicators. **Prepare a series of solutions of varying acidities as outlined in the following table, p. 382. Introduce 5- or 10-ml. portions of each of these into a series of test tubes and to each add a few drops of a solution of thymol blue. Make a note of the colors produced, in the spaces left for this purpose. In the same way test the other indicators mentioned, in order, in each case using a few drops of the indicator solution.**

Are the following assumptions, on which the use of certain of these indicators in gastric analysis is based, borne out by your findings?

1. That Töpfer's reagent (dimethylaminoazobenzene) gives its characteristic pinkish-red color only in the presence of free HCl.

2. That Congo red can be used to distinguish between strong acids, moderately weak acids, and very weak acids.

3. That thymol blue may be used as an indicator in the titration of both free and total acid.

4. That alizarin may be used in titrations where the end point is at a pH just acid to pH 7 rather than just beyond pH 7.

5. That phenolphthalein can be used in titrating total acidity, that is, acidity due to mineral and organic acids, acid salts, and combined acid.

6. That Günzberg's test is in certain respects the most satisfactory one for free HCl.

7. That "combined acid" (protein hydrochloride) is an acid of approximately the strength of acetic acid.

Indicator	*pH Range*	*Color Change*
Thymol blue (acid range)	1.2– 2.8	Red-yellow
Töpfer's reagent	2.9– 4.0	Red-yellow
Bromophenol blue	3.0– 4.6	Yellow-blue
Congo red	3.0– 5.0	Blue-red
Methyl orange	3.1– 4.4	Orange red-yellow
Bromocresol green	4.0– 5.6	Yellow-blue
Methyl red	4.2– 6.3	Red-yellow
Litmus	4.5– 8.3	Red-blue
Chlorophenol red	5.0– 6.6	Yellow-red
Alizarin red	5.0– 6.8	Yellow-red
p-Nitrophenol	5.0– 7.0	Colorless-yellow
Bromocresol purple	5.4– 7.0	Yellow-purple
Bromothymol blue	6.0– 7.6	Yellow-blue
Phenol red	6.6– 8.2	Yellow-red
Neutral red	6.8– 8.0	Red-yellow
Cresol red	7.2– 8.8	Yellow-red
Meta cresol purple	7.6– 9.2	Yellow-purple
Thymol blue (alkaline range)	8.2– 9.8	Yellow-blue
Phenolphthalein	8.3–10.0	Colorless-red
Alizarin yellow	10.0–12.0	Colorless-yellow
Tropaeolin O	11.1–12.7	Yellow-orange

***Special Test for Free HCl* (*Günzberg's Test*). Perform the following test on Solutions 1 to 4 of the table and tabulate the results. Place 1 to 2 drops of Günzberg's reagent[9] in a small porcelain evaporating dish and carefully evaporate to dryness over a low flame. Insert a glass stirring rod into the mixture to be tested and draw the moist end of the rod through the dried reagent. Warm again gently and note the production of a purplish-red color in the presence of free hydrochloric acid. This test differs markedly from the use of indicators in that the reaction is not determined by the pH of the solution, but is based on the fact that hydrochloric acid of any strength whatever reaches a "constant boiling" concentration of about 20 per cent on evaporation. At this strength of acid the ingredients of the reagent condense to form a purplish-red compound. The reaction is thus highly specific for hydrochloric acid in gastric juice.**

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[9] See Appendix.

TABULATION OF RESULTS OF TESTS ON INDICATORS*

Solution	*Approximate Normality or Molarity*	*Approximate pH*	*1* *Thymol Blue* 1.2–2.8 8.2–9.8	*2* *Töpfer's Reagent* 2.9–4.0	*3* *Methyl Orange* 3.1–4.4	*4* *Bromophenol Blue* 3.0–4.6	*5* *Congo Red* 3.0–5.0	*6* *Bromocresol Green* 4.0–5.6	*7* *Chlorophenol Red* 5.0–6.6	*8* *Alizarin* 5.0–6.8	*9* *Bromothymol Blue* 6.0–7.6	*10* *Cresol Red* 7.2–8.8	*11* *Phenolphthalein* 8.3–10.0	*12* *Günzberg's Reagent* (see p. 381)
1. 0.4 per cent HCl..................	N/10	1.0												
2. 0.04 per cent HCl...............	N/100	2.0												
3. 0.6 per cent acetic acid..........	N/10	3.0												
4. 0.04 per cent combined HCl†.....	N/100	4.0												
5. Acid phosphate 9:1‡............	M/15	6.0												
6. Acid phosphate–basic phosphate 4:6‡........................	M/15	7.0												
7. Basic phosphate 20:1‡...........	M/15	8.0												
8. Borate–NaOH 6:4§...............	N/10	10.0												
9. 0.4 per cent NaOH..............	N/10	13.0												

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* Indicator solutions. *Thymol blue, Cresol red, Bromophenol blue, Bromocresol green, Chlorophenol red, and Bromothymol blue*, 0.04 g. in 100 ml. of alcohol. *Methyl orange*, 0.1 g. in 100 ml. of water. *Töpfer's reagent*, 0.5 g. of dimethylaminoazobenzene in 100 ml. of 95 per cent alcohol. *Congo red*, 0.5 g. in 90 ml. of water and add 10 ml. of 95 per cent alcohol. *Alizarin*, 1 g. of sodium alizarin sulfonate in 100 ml. of water. *Phenolphthalein*, 1 g. in 100 ml. of 95 per cent alcohol.

† Combined HCl. Treat 0.4 per cent HCl with a small amount of Witte's peptone and boil until the solution no longer gives a blue but only a brown color with Congo-red paper.

‡ Make up solutions of potassium dihydrogen phosphate and of disodium hydrogen phosphate of M/15 strength. See p. 35. To prepare the acid phosphate solution used in the test, mix 1 part of the solution of the disodium salt with 9 parts of the solution of the dihydrogen phosphate. For the basic phosphate solution the proportions are 20:1; for the solution of pH 7, the proportions are 6:4. Do not attempt to use distilled H_2O alone as a pH 7 solution. (Why not?)

§ Borate-NaOH solution. Prepare a borate solution by dissolving 12.404 g. of pure boric acid (0.2 mol.) in 100 ml. of N NaOH solution and dilute with water to a liter. Prepare the borate-NaOH solution by mixing 6 parts of the borate solution with 4 parts of 0.1 N NaOH.

Differential Titration of a Mixture of Strong and Weak Acids. (_a_) Titrate a 5-ml. portion of 0.1 N acetic acid with 0.1 N sodium hydroxide, using phenolphthalein as indicator. (_b_) Titrate a 5-ml. portion of 0.1 N hydrochloric acid, using phenolphthalein. (_c_) Titrate a 5-ml. portion of 0.1 N hydrochloric acid, using Töpfer's reagent (one drop) as indicator. The end point is a salmon-pink shade, intermediate between red and yellow. Is there any significant difference in the titer of the hydrochloric acid as compared with the value obtained in (_b_)? (_d_) Now mix 5-ml. portions of 0.1 N acetic acid and 0.1 N hydrochloric acid in a flask, add Töpfer's reagent, and titrate with alkali to the salmon-pink end point. Read the buret, add a drop of phenolphthalein to the contents of the flask, and continue the titration with alkali until the pink color of the phenolphthalein end point can be seen to be superimposed on the clear yellow color of the Töpfer's reagent. Read the buret again. This reading represents the "total acidity." The first reading is a measure of the HCl present, and the difference between the first and final readings represents the acidity due to the weak acid (acetic, in this case). Compare the values you obtain in this differential titration with those obtained by separate analysis in parts (_a_) and (_c_) above.

THE FRACTIONAL METHOD OF GASTRIC ANALYSIS

Since 1914, when the first experiments entailing the use of the fractional method were reported from the senior author's laboratory, the method has been widely adopted both in this country and abroad. This widespread use by a large number of workers has resulted in much discussion of the method. On the basis of this large experience certain modifications in the original technique are here presented. In its major details, however, the method remains as originally carried out, and in the opinion of the authors constitutes the best available method for the clinical examination of the stomach.

When an Ewald test meal is given to normal individuals a curve such as Curve (1) in Fig. 95 is usually obtained. The curve may vary within certain limits depending on individual idiosyncrasies, but it is usually found to follow the curve depicted, and the meal normally leaves the stomach in two and one-half hours. Pathologically every variation occurs, in time of evacuation as well as in the character of the curve and the quantity of the secretion elaborated. Fig. 95 represents some of the possibilities of pathological cases, but a consideration of their interpretation is outside the purpose of the present volume. It is evident, however, that the cycle of gastric digestion is a constantly changing one, and no information concerning the trend of digestion can be obtained by an examination at only a single stage of digestion. Marked changes may precede or follow that stage.

Low acidities may be found in carcinoma, in atonic dyspepsia and in pernicious anemia. However, anacidity of itself is not necessarily of pathological significance. A tendency toward high acidities may be found, though not constantly, in cases of gastric and duodenal ulcers, especially those occurring in the neighborhood of the pylorus and inducing some degree of obstruction. High acidities may be induced reflexly in gallbladder disease and appendicitis.

As this is written, one of the favorite means used to combat the high acid tendency of ulcer is the chemical substance called _Banthine_ (β-diethylaminoethylxanthene-9-carboxylate methobromide).[10] However, there are those who do not believe that Banthine is the final answer to the ulcer problem.[11] It is suggested that the drug should be used to supplement conventional methods of treatment rather than to replace them. Pro-banthine and Prantal have also been recommended for use in ulcer therapy. In the past, roentgen therapy as well as the use of radioactive phosphorus (P^{32}) and radioactive iodine (I^{131}) have been used with indifferent success to suppress gastric acidity clinically. *Radioactive krypton* may possibly prove more effective for this purpose.[12] However this has not been definitely proved.

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[10] Benjamin, Rosiere, and Grossman: _Gastroenterology_, **15**, 727 (1950).

[11] Ruffin: _Gastroenterology_, **17**, 589 (1951); Levin, Kirsner, and Palmer, _Gastroenterology_, **21**, 339 (1951).

Some gastroenterologists have found the *gastrophotor* useful in the study of ulcer. This is a tiny camera which may be swallowed by the patient. By its use 16 pictures of various sections of the stomach walls may be taken at the same time.

OBTAINING THE SAMPLES

1. Introduction of the Stomach Tube. Whereas the large tube is directly inserted by propulsion, the Rehfuss tube is swallowed in the natural manner with the aid of gravity. The tube may be passed in one of three ways, viz., (1) lubricated; (2) with the aid of fluid; (3) after the throat is cocainized. When passed by the first method the tip of the tube, after thorough lubrication with glycerol or liquid petrolatum is held between the thumb and forefinger and placed on the tongue. Then with the aid of the forefinger the tip is pushed backward until it reaches the root of the tongue and is engaged in the oropharynx. Then the patient is encouraged to breathe deeply through the nose and to swallow persistently while the tube is slowly fed into the mouth. After slight discomfort in the pharynx and its passage past the level of the cricoid cartilage, practically no discomfort is felt. This method is used when it is essential that the pure gastric secretion or residuum be obtained. Ordinarily, however, it is much easier to swallow the tube by the second method. This method consists in placing the tip in the oropharynx and then giving the patient a measured quantity of water or tea to swallow. The movements induced by the swallowing carry the tube rapidly to the stomach with a minimum of discomfort. When an Ewald meal (see p. 385) is given, part of the tea may be reserved for swallowing the tube. This procedure makes it scarcely more arduous than the swallowing of food. Should the patient, however, be extremely neurotic or the unfortunate possessor of marked pharyngeal hyperesthesia, cocaine hydrochloride in 2 per cent aqueous solution may be applied to the throat, rendering the passage of the tube practically insensible. When the tube has entered the stomach, aspiration of the material shows the characteristic gastric contents. Should the tip remain in the esophagus through transient cardiospasm or other cause, aspiration results in the removal of only a very small specimen having all the characteristics of the pharyngeal and esophageal secretions.

The clinician should see to it that the tube is so placed that the tip reaches the lower pole of the stomach. Furthermore, *the tip should remain in this position throughout the duration of the test*. By placing the tube in this manner, representative specimens and accurate data may be obtained.

It has been shown that the gastric response to the fractional method when properly performed is similar for the same individual on different days[13]

Bloomfield and Keefer have suggested a method for the "continuous quantitative estimation of gastric secretion and discharge in man."

2. Removal and Analysis of Residuum. If the so-called empty stomach is examined in the morning before any food or drink has been taken it will be found to contain considerable material, which is termed *residuum*. Before a test meal is introduced into the stomach, this organ should be emptied. If this is not done we cannot consider the samples withdrawn after the test meal is eaten as representing the secretory activity of the gastric cells under the influence of the stimulation of the test meal. It has been generally recognized, clinically, that a residuum greater than 20 ml. is pathological. Such a volume has been considered as indicative of hypersecretion, and this in turn in many cases indicates an organic lesion. The observations indicating that a residuum of over 20 ml. was pathological were made upon residuums removed by means of the old type of stomach tube which does not completely empty the stomach. When the residuum is completely removed by means of the Rehfuss tube it has been demonstrated that the *normal* residuum is practically always over 20 ml., the average for both men and women being about 50 ml. The normal residuum has been found to possess all the qualities of a physiologically active gastric juice with an average total acidity of 30 and an average free acidity of 18.5. The residuum is often colored by bile. This is particularly true if the fluid has a relatively high acidity. Trypsin is also generally present. These findings indicate regurgitation (see p. 390). Pathological residuums may contain blood, pus, and mucus and may also show food retention, indicative of delayed evacuation. In carcinoma the residuum frequently has a foul odor. The quantity may also be much increased owing to hypersecretion. A residuum of large volume possessing a total acidity value of 70 or over may indicate ulcer.

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[12] Steinfield: *Proc. Soc Exptl. Biol. Med.*, **81**, 636 (1952).

[13] Ryle: *Gastric Function in Health and Disease*, London, Oxford University Press, 1926.

Remove the residuum as directed under (5) on p. 386, and analyze the fluid according to methods outlined on p. 386.

3. Feeding the Test Meal. Before making an analysis of the stomach contents it is customary to introduce something into the stomach which will stimulate the gastric cells. The response to this stimulation is then measured clinically by the determination of total acidity, free acidity, and pepsin in the stomach contents. Many forms of test meal have been used.

The test meal most widely employed is the Ewald test meal. This consists of 2 pieces (35 g.) of toast and 8 ounces (250 ml.) of tea.

Inasmuch as it was demonstrated in the senior author's laboratory that water gave a similar gastric stimulation to that produced by the Ewald meal, it was suggested that a simple water meal might be substituted for the Ewald meal. This water meal also has the added advantage of enabling one to determine the presence of food rests and to test more accurately for lactic acid, blood, and bile. Ryle[14] prefers to use oatmeal gruel (1 pint). Another test meal favored in certain English hospitals consists of 20 g. citrus pectin, 35 g. sucrose, 60–70 mg. phenol red and sufficient caustic soda to raise the pH to 6.5 in one liter of distilled water.[15] The alcohol test meal has also been suggested.[16] This consists of 200 ml. of 5 per cent ethyl alcohol. Doses of 0.5 to 1 mg. of histamine hydrochloride injected subcutaneously are effective in stimulating gastric secretion and may help to differentiate between true and "apparent" achylia gastrica.

4. Feeding the Retention Meal. In order to obtain more information regarding gastric motility than is furnished by the ordinary test meal described above, the patient may be fed a so-called retention meal. This meal is fed in place of the regular evening meal and contains substances readily detected.

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[14] Boil 2 tbsp. of oatmeal in a quart of water until the total bulk is reduced to 1 pint. Strain through coarse muslin. Season with salt as desired.

[15] Hunt: *Lancet*, **257**, 794 (1949), and *J. Physiol.* (*London*), **113**, 169 (1951).

[16] Ehrmann: *Am. J. Digestive Diseases*, **13**, 23 (1946).

In the morning before breakfast (7 to 8 A.M.), remove the stomach contents (residuum, see p. 384) by aspiration and examine for food rests. The normal stomach should give no evidences of food retention. A satisfactory retention meal consists of 4 ounces each of boiled string beans and rice. Diets containing prunes, raspberry marmalade, lycopodium powder, etc., have also been employed. In many instances an ordinary mixed diet will serve the purpose.

5. *Removal of Samples of Stomach Contents.* At intervals of exactly 15 minutes from the time the test meal is eaten until the stomach is empty, 5- to 6-ml. samples of gastric contents are withdrawn from the stomach by means of aspiration. (A few milliliters of air is blown down the tube in order that the tube shall be empty when the next sample is drawn.) If the stomach is not empty at the end of three hours, the remaining stomach contents may be withdrawn and measured.

In order to facilitate the mixing of the stomach contents and the withdrawal of a more representative sample, the stomach contents should be aspirated back and forth four times before taking the sample for analysis. Some clinicians advise that the stomach contents be mixed by physical manipulation just prior to aspiration.

In the removal of samples from the stomach, it is essential that very little traction be employed. To completely empty the stomach, aspiration is practised in four positions: (*a*) on the back, (*b*) on the stomach, (*c*) on the right side, and (*d*) on the left side. This results in complete evacuation of the stomach. Three tests may be employed to determine whether the stomach is empty: (1) No more material can be aspirated in any position. (2) Injection of air and auscultation over the stomach with a stethoscope reveals a sticky rale and not a series of gurgling rales such as is heard when there is material in the stomach. (3) Lavage or irrigation through the tube shows the absence of all food in the stomach.

EXAMINATION OF THE SAMPLES

The old methods of gastric analysis involved the collection (by analysis and calculation) of data regarding several types of acidity. It has already been pointed out (p. 380) that the validity of such data is questionable. The modern tendency among clinicians is to lay particular emphasis upon the values for total acidity, free acidity, and pH. Chloride partition may also give some information. The determination of the peptic activity is also of occasional value as well as the demonstration of the presence or absence of occult blood, lactic acid, mucus, food rests, etc.

Strain each sample through fine-mesh cheesecloth.[17] Examine the residue for mucus, blood,[18] and food rests. Use the strained stomach contents for the determination of total acidity, free acidity, and peptic activity by methods which follow.

1. *Determination of Total Acidity. Principle.* The indicator used is phenolphthalein. Since the indicator reacts with mineral acid, organic acid, combined acid, and acid salts, the values obtained represent the *total acidity* of the solution.

***Procedure.* Measure 1 ml. of the strained stomach contents by means of an Ostwald pipet and introduce it into a low-form 60-ml. porcelain evaporating**

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[17] Any examination required for microscopic constituents should be made on the original (unstrained) gastric contents. Tests for occult blood may be made on the sediment if desired.

[18] The detection of blood is rather more satisfactory in the residue than in the strained fluid.

dish. Dilute with 15 ml. of distilled water. Add 2 drops of a 1 per cent alcoholic solution of phenolphthalein and titrate with 0.01 N sodium hydroxide until a faint pink color is obtained which persists for about 2 minutes. Take the buret reading and calculate the total acidity.

CALCULATION. Note the number of milliliters of 0.01 N NaOH required to neutralize 1 ml. of stomach contents, and multiply it by 10 to obtain the number of milliliters of 0.1 N NaOH necessary to neutralize 100 ml. of stomach contents. This is the method of calculation most widely used.

2. Determination of Free Acidity: Principle. An indicator is selected which changes color at sufficiently low pH values so that the end-point corresponds to the neutralization of all highly ionized acid only, as discussed on p. 378. The indicator most widely used is Töpfer's reagent; its advantages and disadvantages have been presented on p. 379. The use of Sahli's reagent, a mixture of potassium iodide and iodate which liberates free iodine in the presence of a sufficiently high concentration of hydrogen ions, has been suggested in place of Töpfer's reagent. The liberated iodine is titrated with thiosulfate solution and the end point is very sharp. Unfortunately, Sahli's reagent reacts to a certain extent with weak organic acids so that high values are obtained for example after the ingestion of acid fruits. A procedure using Sahli's reagent is given in the eleventh edition of this book.

Procedure. **By means of an Ostwald pipet measure 1 ml. of the strained stomach contents and introduce it into a low-form 60-ml. porcelain evaporating dish. Dilute with 10 ml. of distilled water. Add 2 drops of Töpfer's reagent (0.5 per cent alcoholic solution of dimethylaminoazobenzene) and titrate with 0.01 N sodium hydroxide to a salmon-pink color. If the sample gives a yellow color on the addition of Töpfer's reagent, it has no free acid present. The end point may require practice in identification. The use of comparative buffered colorimetric standards to control the end point has been suggested.[19] Care should be taken against the possibility of color fading. The reading of the buret at the end point is a measure of the free acidity.**

It is common clinical practice to use this same sample for the determination of total acidity. To do this, add 2 drops of a 1 per cent alcoholic solution of phenolphthalein, and continue the titration until the pink color change of the phenolphthalein is superimposed on the yellow color of the Töpfer's reagent. The buret reading at this point is a measure of the total acidity.

CALCULATION. Note the number of milliliters of 0.01 N sodium hydroxide required to reach the end-point color with Töpfer's reagent. Multiply this by 10 to obtain the number of milliliters of 0.1 N sodium hydroxide necessary to neutralize the free acid in 100 ml. of gastric contents. This value also corresponds to the free acidity expressed in degrees or in milliequivalents of acid per liter. In a similar way, the buret reading at the phenolphthalein end point can be used to calculate the total acidity.

3. Determination of Hydrogen-ion Concentration of Gastric Contents. Principle. Inasmuch as the hydrogen-ion concentration of the gastric contents is a determining factor in peptic digestion the estimation of pH is coming into wider use in gastric analysis. Simple colorimetric methods are available for this purpose, as is the convenient electrometric pH meter. The measurement of pH gives information of real value and the test-paper method especially is so convenient that it may often be the method of choice.[20] Determinations of pH do not, however, displace titration methods, since the latter give additional information.

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[19] Berk, Thomas, and Rehfuss: *Am. J. Digestive Diseases*, **9**, 106 (1942).

[20] Prepared outfits for both the indicator solution and test paper methods may be obtained from most larger laboratory supply houses.

(a) COLORIMETRIC METHOD OF SHOHL AND KING.

Procedure. Prepare Clark and Lubs standard solutions for pH 1.4, 1.6, 1.8, 2.0, 2.4, 3.0 (see Chapter 1). Transfer 2 ml. of filtered or centrifuged gastric contents to a test tube 11 mm. in diameter. Add 2 drops or 0.04 ml. of 0.2 per cent thymolsulfonephthalein in 95 per cent alcohol. Compare with equal amounts of standard solutions treated in the same way in similar test tubes. If the sample is more acid than pH 1.4, lower standards may be prepared. Using the Ewald test meal it may be said in a general way that pH 1.4 represents a high acidity; from 1.6 to 1.8, moderate normal acidity; from 2.0 to 2.4, low acidity; and 3.0 or higher, anacidity.

(b) METHOD OF DENIS AND SILVERMAN USING TEST PAPERS.

Procedure. Prepare test papers as follows. A solution of dye (dimethylaminoazobenzene 0.5 per cent or 0.1 per cent thymol blue) is poured over a sheet of filter paper six inches in diameter, the excess dye allowed to drain off, and the paper dried quickly by holding it in front of a rapidly revolving electric fan. Prepare the two kinds of paper, cut in strips approximately 1 by ¼ inch, and place in a stoppered bottle. Keep away from light and laboratory fumes. Smooth, ashless quantitative paper of the highest grade should be used.

Pour some of the material to be tested over the end of a test paper. The colors may be compared with those obtained from similar papers dipped in the standard solutions mentioned in the method of Shohl and King above. However, with some practice this will be unnecessary. All readings should be made at once. The table given below will serve as a guide in the interpretation of results. It gives test-paper results as compared with number of ml. of 0.1 N NaOH that would be required to neutralize 100 ml. of a solution of the H-ion concentration found, if all the acid were free HCl.

Indicator	*pH*	*Color*	*Ml. 0.1 N NaOH Required*	*Remarks*
Töpfer's reagent	1.4	Deep red	44	Hyperacidity
Töpfer's reagent	1.6	Reddish-orange	27	Normal acidity
Töpfer's reagent	1.8	Orange	17	Normal acidity
Töpfer's reagent	2.0	Yellowish-orange	10	Hypoacidity
Thymol blue	2.0	Purple-red	10	Hypoacidity
Thymol blue	2.4	Faint pink	4	Hypoacidity
Thymol blue	3.0	Yellow	1	Anacidity

4. *Determination of Peptic Activity.*

(a) PHOTOMETRIC METHOD OF RIGGS AND STADIE. In this method the enzyme activity is measured photometrically as the decrease in turbidity of a standardized, homogenized suspension of coagulated egg white under specified conditions. Accurate measurement is possible down to a level equivalent to 1 μg. of crystalline pepsin. Protein hydrolysis so measured follows a monomolecular course and hence the enzyme activity is expressed as a velocity constant. This method is particularly adapted to clinical studies. For details of procedure see the original article.[21]

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[21] Riggs and Stadie: *J. Biol. Chem.*, 150, 463 (1943).

(b) Hemoglobin Method of Anson and Mirsky. Principle. The pepsin is added to a hemoglobin solution. Unaltered protein is removed with trichloroacetic acid. The amount of digestion products is determined colorimetrically, using phenol reagent, which reacts with tyrosine, tryptophan, and cysteine groups, tyrosine being used as a standard.

For a discussion of the determination of pepsin by viscosimetric and other methods and of pepsin units see Northrop.[22]

Procedure. **Pipet 5 ml. of a 2 per cent solution of dialyzed ox carbon monoxide hemoglobin[23] in 0.06 N HCl into a 175 by 20 mm. test tube and bring to 35.5° or 25° C. Add 1 ml. of enzyme solution and mix by whirling tube. After 5 minutes add 10 ml. of 4 per cent trichloroacetic acid from another test tube and pour back and forth to mix. Filter through fine paper. To 3 ml. of filtrate in a 50-ml. Erlenmeyer flask add 20 ml. of water, 1 ml. of 3.85 N NaOH, and 1 ml. of phenol reagent (Folin and Ciocalteu. See Chapter 31). The standard consists of 3 ml. of 0.1 N HCl containing 0.15 mg. of tyrosine. (A copper sulfate standard can be used with a red color filter.) Compare colors after 5 to 10 minutes with the standard at 20.**

Calculation.[24] If X is the reading of the unknown:

$$\text{Pepsin Units} = (0.0194/X) - 0.000147$$

If carried out at 25° C. multiply by 1.82.

(c) Method of Volhard and Löhlein. Principle. The pepsin is added to casein solution. The unaltered casein is salted out. The filtrate from this contains the digestion products of casein which can be estimated by titration.

Procedure. **Into each of three flasks (graduated at 300 and at 400 ml.) introduce 11 ml. of N HCl and add water to make not quite 150 ml. With con-**

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[22] Northrop: *J. Gen. Physiol.*, **16**, 41 (1932).

[23] Bubble CO through whipped ox blood. Centrifuge. Siphon off the serum and cells. Wash the corpuscles four times with cold 0.9 per cent NaCl. Add an equal volume of water and a sixth of the total volume of toluol. Bubble the CO through the solution. Shake vigorously. Let stand in the cold over night. Siphon off the hemoglobin layer. Mix gently with a tenth the volume of centrifuged alumina cream (see Appendix). Filter through coarse paper. Dialyze over night in the cold and keep under CO in the cold with toluol as a preservative. Determine N by the Kjeldahl method. Use 17.7 as per cent of N in the hemoglobin. Use mercury instead of copper in the determination and evaporate before digestion to avoid serious foaming. To 3 volumes of 0.1 N HCl add 2 volumes of 5 per cent hemoglobin. This solution may be kept in the cold for 10 days.

The tyrosine used should have been recrystallized and the concentration also determined by the Kjeldahl method. N = 7.74 per cent.

[24] In addition to the 0.15 mg. of tyrosine the standard contains from the reagents an amount of color-producing material equivalent to 0.015 mg. of tyrosine. The color-producing substance in the 3 ml. of filtrate is, therefore, equivalent to $(20/X)$ $(0.15 + 0.015)$ mg. of tyrosine. Of this 0.015 mg. are due to the color-producing substance in the reagents and 0.01 mg. to the color-producing substance present in the trichloroacetic acid filtrate even when no enzyme is added. The digested hemoglobin in the 3 ml. of filtrate is, therefore, equivalent to $(20/X)$ $(0.15 + 0.015) - 0.015 - 0.01$ mg. of tyrosine. This value must be multiplied by $^{16}/_{3}$ to obtain the digested hemoglobin in the whole 16 ml. of filtrate instead of the 3 ml. taken for analysis: it must be divided by 5 to obtain the amount of nonprecipitable digested hemoglobin produced in one minute instead of in 5 minutes; it must be divided by 181, the molecular weight of tyrosine, to obtain the tyrosine equivalent as milliequivalents instead of as milligrams. The relation between the number of pepsin units in 1 ml. of enzyme solution and the colorimeter reading X is thus:

$$P.\,U. = \left[\frac{20}{X}(0.15 + 0.015) - 0.015 - 0.01\right]\frac{16}{3} \times \frac{1}{5} \times \frac{1}{181} = \frac{0.0194}{X} - 0.000147$$

stant shaking add 100 ml. of the casein solution.[25] **Any turbidity must quickly disappear. Add different known amounts of pepsin-containing solution to the three flasks and make up to 300 ml. Keep at 40° C. for one hour. Add 20 per cent sodium sulfate solution to the 400-ml. mark. Filter and titrate 100 ml. of filtrate with 0.1 N NaOH, using phenolphthalein as an indicator.**

CALCULATION. Multiply the titration by 4. Correct for blank run without pepsin and for any acidity of the pepsin solution. The square of the result gives units of pepsin. In any pepsin method it is best to determine the amount of unknown required to bring about, in a definite time, the same amount of digestion as a definite amount of a standard solution of pepsin.[26]

5. *Determination of Tryptic Activity.* Trypsin is not a gastric enzyme but occurs in the pancreatic juice (see p. 395). In case of regurgitation of intestinal contents through the pylorus, trypsin would be passed into the stomach. The regurgitation is doubtless of frequent occurrence and may even be a normal mechanism by which gastric acidity is regulated (see p. 378). Trypsin is, therefore, generally present in the contents of the normal human stomach. However, inasmuch as trypsin is destroyed by the pepsin–hydrochloric acid of the gastric juice, determinations of this enzyme must be carried out immediately after aspirations of the gastric contents, particularly where the acidity of the latter is high.

SPENCER'S METHOD.[27] **(*a*) Prepare five reagent tubes, Nos. 1, 2, 3, 4, and 5; more if desired.**

To Tubes 1 and 2 add 0.5 ml. of gastric contents (filter if cloudy).

(*b*) To Tubes 2, 3, 4, and 5 add 0.5 ml. of distilled water.

(*c*) From Tube 2 remove 0.5 ml. of its mixed contents and add to Tube 3. Mix thoroughly and add 0.5 ml. from Tube 3 to Tube 4. Repeat for Tube 5. We now have dilutions of gastric contents of 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, and $\frac{1}{16}$.

(*d*) To each tube add one drop of phenolphthalein solution (phenolphthalein 1 g.; alcohol (95 per cent) 100 ml.); then drop by drop add a 2 per cent sodium bicarbonate solution until a light pink color is produced.

(*e*) To Tubes 1, 2, 3, and 4 add 0.5 ml. of casein solution. Tube 5 must receive 1 ml. of casein solution, since it contains 1 ml. of the diluted gastric contents. For the casein solution, dissolve 0.4 g. of casein in 40 ml. of 0.1 N NaOH. Add 130 ml. of distilled water, then 30 ml. of 0.1 N HCl. This leaves the solution alkaline to the extent of 10 ml. of 0.1 N NaOH, minus about 3 ml. neutralized by the casein.

(*f*) Incubate for five hours at 40° C.

(*g*) Precipitate the undigested casein by the dropwise addition of a solution of the following composition: glacial acetic acid 1 ml., alcohol (95 per cent) 50 ml., distilled water 50 ml. The tubes in which digestion has been complete remain clear; others become turbid.

(*h*) The tryptic values are expressed in terms of dilution. Thus, complete digestion in Tube 3 (a dilution of $\frac{1}{4}$) shows four times the tryptic power of undiluted gastric juice; taking this dilution as unity, the undiluted juice has a tryptic value of 4.

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[25] Introduce 100 g. of pure casein into a 2-liter flask. Add 1000 ml. of water, shake, and let stand some hours. Add 80 ml. of N NaOH, make up to 2000 ml., and warm gradually until clear. Then heat rapidly to 85°-90° C. to destroy any proteinases and preserve in a stoppered bottle with a little toluol. This solution keeps for a long time.

[26] Treat 10 g. of a good pepsin preparation with 100 ml. of 10 per cent NaCl. Let stand one week at room temperature. Filter. Add an equal volume of glycerol and keep in the ice box. The solution keeps indefinitely.

[27] Elaborated by Spencer in the senior author's laboratory for the specific purpose of determining trypsin in gastric juice.

(*i*) Controls of boiled gastric contents plus casein solution, and of distilled water plus casein solution, treated as above stated, must show no digestion, and become turbid on addition of the precipitating solution.

6. Detection of Lactic Acid. When the acidity of the stomach contents is reduced to a low value there may occur considerable fermentation of carbohydrates which have been introduced into the stomach in the ingested food. This fermentation yields various organic acids, among which lactic acid is particularly prominent. It is important, therefore, in case of low gastric acidity that the stomach contents be examined for lactic acid.

ETHER–FERRIC CHLORIDE TEST (STRAUSS). A satisfactory deduction regarding the presence of lactic acid can be made only by removing the lactic acid from interfering material (e.g., hydrochloric acid, protein digestion products, etc.) present in the stomach contents. Lactic acid may be extracted from the stomach contents by ether. The following technique not only serves to detect lactic acid but also gives an approximate idea as to the amount of the acid present.

***Procedure.* Introduce 5 ml. of strained stomach contents into a small graduated separatory funnel, add 20 ml. of ether, and shake the mixture thoroughly. Permit the ether to separate, then allow all the fluid to run out of the separatory funnel except the upper 5 ml. of ether. To this ether extract add 20 ml. of distilled water and 2 drops of a 10 per cent solution of ferric chloride and shake the mixture gently. A slight green color is obtained in the presence of 0.05 per cent lactic acid whereas 0.1 per cent lactic acid yields a very intense yellowish-green color.**

7. Detection of Occult Blood.[28] BENZIDINE REACTION. **This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light it is essential that they be kept in a dark place. The test is performed as follows: To 3 ml. of a saturated solution of benzidine in glacial acetic acid[29] add 2 ml. of the solution to be tested and then 1 ml. of 3 per cent hydrogen peroxide. A positive test is indicated by a green or blue color.**

CONFIRMATORY TEST. **If the mixture contains fat, make neutral or slightly alkaline, and extract by shaking with an equal volume of ether. Discard this ether extract. Make the residue acid with acetic acid and again extract with ether. Pour off ether into a small evaporating dish. Put on a hot water bath with flame turned out and evaporate to dryness. Add a few drops of water, a drop of benzidine solution, and a drop of 3 per cent hydrogen peroxide. A green or blue color indicates blood.**

Blood is found in gastric contents in conditions associated with erosion of the mucous membrane, ulcer, and carcinoma. In cases of ulcer the blood may be bright red or may be converted to brown "acid hematin" by the excess of acid which is usually present. In carcinoma the blood forms brownish-black lumps, the so-called "coffee-ground" material.

8. Detection of Bile in Stomach Contents. If we accept Boldyreff's theory as to the automatic regulation of gastric acidity under normal conditions by the regurgitation of alkaline material from the intestine, then the presence of bile in the gastric juice does not possess the clinical significance it has been accorded. However, if an ordinary Ewald meal be fed, and bile in any considerable quantity be found throughout the entire course of digestion, it may indicate, pathologically, a stenosis below the level of the common bile duct. The presence of bile is indicated by a yellowish or greenish color of the specimen, changing to a bright green on standing.

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[28] These tests may be made upon the strained stomach contents or upon the solid residue.

[29] Glacial acetic acid is preferable, but alcohol acidified with acetic acid may be used.

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16

Pancreatic Digestion

As soon as the food mixture leaves the stomach it comes into intimate contact with the bile and the pancreatic juice. Since these fluids are alkaline in reaction and have high buffering qualities, there can obviously be little further peptic activity after they have become intimately mixed with the chyme and have neutralized most of the acidity previously imparted to it by the hydrochloric acid of the gastric juice. The pancreatic juice reaches the intestine through the duct of Wirsung which usually joins with the common bile duct before opening into the intestine near the pylorus.

STIMULATION OF PANCREATIC SECRETION

Secretin and Pancreozymin. Prior to the work of Bayliss and Starling in 1902 it was believed that substances in the intestine stimulated pancreatic secretion through local reflexes from the intestine to the pancreas. This view was based on the observation that the introduction of acid into the intestine was followed by secretion. Bayliss and Starling, however, observed that introduction of acid into a jejunal loop which had been denervated as far as possible still induced secretion. This indicated that the exciting agent acted by way of the blood. Injection of acid directly into the blood did not affect secretion, which must therefore be induced by some substance given off from the mucosa. A substance capable of stimulating secretion by intravenous injection could be extracted from the intestinal mucosa by acid but not by water. Apparently the mucosa is stimulated by the acid of the gastric chyme to liberate *secretin* which passes by way of the blood stream to the pancreas causing it to secrete. This view has been confirmed by many observers, and most clearly by Ivy, Farrel, and Lueth, who found acid applied to transplanted intestinal loops to cause secretion in the transplanted pancreas. The fact that secretin is more readily extractable from the intestinal mucosa by acid than by neutral solvents was interpreted by Bayliss and Starling to mean that the hormone exists in the mucosa as prosecretin. It seems more likely, however, that secretin exists preformed and that the acid chyme serves to release it from its adsorbed state on the colloidal components of the mucosa.

Secretin was the first hormone to have its function clearly established. Tests on the purest secretin preparation so far obtained show it to be a polypeptide, with generally basic properties. Edman and Ågren have demonstrated the existence of 15 (or 16) amino acids in secretin, and quantitative data are available for seven of these. Although earlier ultracentrifugal measurements indicated a molecular weight of 5000, the values for total sulfur and cystine suggest a figure twice this. The activity of secretin is destroyed by pepsin and trypsin; hence it cannot be given effectively by mouth. Secretin is likewise destroyed on incubation with blood serum, presumably because of the presence of the enzyme secretinase.

Harper and Raper[1] first determined that extracts of duodenal mucosa contain *two* hormonal agencies affecting the external secretory activity of the pancreas. These are (1) secretin, which stimulates the production of fluid and bicarbonate by the pancreatic acini, and (2) pancreozymin, which governs the production of the pancreatic enzymes. Pancreozymin stimulates the output of all pancreatic enzymes, and when injected repeatedly, sustains high enzyme concentrations for prolonged periods. Like secretin, pancreozymin is inactivated by blood serum. Using dogs with a part of the pancreas subcutaneously autotransplanted, Wang and Grossman[2] have observed that products of protein digestion (peptone and amino acids) are most effective in stimulating release of pancreozymin; next most effective are soaps (sodium oleate) and, to a lesser extent, HCl. Carbohydrates are ineffective.

It might be supposed that a failure of the pancreatic secretion would be noted in achylia, in which condition no gastric acid is secreted to bring about the liberation of secretin. This is not the case. Fatty acids formed in fat digestion and protein split products may cause the passage of secretin into the blood stream. For the same reason bile also has some stimulating action on pancreatic secretion.

PANCREATIC JUICE

The juice as obtained from a permanent fistula differs greatly in its properties from the juice obtained from a temporary fistula, and neither form of fluid possesses the properties of the normal fluid. Pancreatic juice collected from a natural fistula has been found to be a colorless, clear, alkaline fluid (pH 8 or thereabouts) which foams readily. The inorganic salts consist largely of sodium chloride and bicarbonate, in approximately equal amounts; the bicarbonate, obtained in part from blood but largely from metabolic processes within the pancreas,[3] is responsible for the alkalinity of the juice. It is further characterized by containing albumin, globulin, proteose, and peptone; nucleoprotein is also present in traces. The average daily secretion of pancreatic juice is 650 ml. and its specific gravity is 1.008. The fluid contains 1.3 per cent of solid matter and the freezing point is −0.47° C.

The normal pancreatic secretion contains a variety of different enzymes. Among those which have been well characterized are included the following: (1) The peptide-splitting enzymes trypsin, chymotrypsin, and various carboxypolypeptidases; (2) a polynucleotidase (or nuclease) acting to split nucleic acids into their component mononucleotides; (3)

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[1] Harper and Raper: *J. Physiol.*, **102**, 115 (1943).

[2] Wang and Grossman: *Am. J. Physiol.*, **164**, 527 (1951).

[3] Hollander and Birnbaum: *Trans. N. Y. Acad. Sci.*, **15**, 56 (1952).

pancreatic amylase (amylopsin), an amylolytic enzyme; and (4) pancreatic lipase (steapsin), a fat-splitting enzyme. Other enzymes are doubtless present. It will be noted that pancreatic juice contains enzymes capable of acting on all three classes of foodstuffs—proteins, carbohydrates, and fats. Ivy and co-workers have reported, however, that the concentrations of these enzymes in pancreatic juice can be markedly influenced by diet. Thus, animals receiving a high protein diet secrete a juice especially rich in trypsin; on a high carbohydrate intake the pancreatic amylase concentration is greatly increased; on a high fat–low protein diet, however, the secretion of all enzymes is inhibited. It has been suggested that *carbonic anhydrase* is importantly related to the intracellular conversion of CO_2 to H_2CO_3 and hence to the formation of HCO_3^- for secretion.[3]

Proteolytic Enzymes of Pancreatic Juice. The major proteolytic enzymes of pancreatic juice appear to be (1) trypsin, (2) chymotrypsin, and (3) a carboxypolypeptidase, each of which has been obtained in crystalline form. The combined activities of these (and possibly other) enzymes was formerly believed to be due to a single enzyme which was called trypsin. This term is now, however, applied to a single enzyme in the group. As a class, these enzymes are similar in that they all catalyze the hydrolytic splitting of the peptide bond. They differ from one another in such respects as the type of peptide linkage required for activity; the type of activation from zymogen precursors, as discussed below; the pH optima, etc.

Trypsin and chymotrypsin may be classified as *endopeptidases* (Bergmann) acting on peptide linkages either in the central portion or the terminal portion of polypeptide chains. By the use of synthetic peptide substrates, Bergmann and Fruton found that trypsin acts on peptide linkages containing the carboxyl group of either lysine or arginine. Chymotrypsin on the other hand was found to act on peptide linkages involving the carboxyl group of tyrosine and phenylalanine. Thus the digestive action of trypsin and chymotrypsin on proteins appears to involve the splitting of specific types of peptide linkages in the molecule, the products of the action being, as with pepsin (see p. 364), either low-molecular-weight polypeptides or free amino acids, depending upon the location of the peptide linkages acted upon. There is some evidence of a synergistic action of these various endopeptidases; the action of one may uncover a linkage previously inaccessible to another.

Trypsin has its greatest activity at pH 8 to 9, the optimum pH depending somewhat on the nature of the substrate. It has some action in weakly acid solution. In acid solutions it resists temperatures near the boiling point, the denaturation that occurs being rapidly reversible on cooling. Trypsin is fairly stable in acidities as high as pH 2, but is digested by pepsin in acid solutions.

The carboxypolypeptidase activity of pancreatic juice is doubtless due to a mixture of enzymes, of which only one has been crystallized (Fig. 97). Carboxypolypeptidase is an example of an *exopeptidase*. It catalyzes the splitting of a peptide linkage involving the amino group of an amino acid whose carboxyl group is free, i.e., not combined in peptide linkage with another amino acid. The action of carboxypolypeptidase is thus confined to the terminal portion of a polypeptide chain. If the free carboxyl group is blocked, as by the formation of an ester, the enzyme becomes ineffective. Carboxypolypeptidase has a pH optimum around 7.0.

ACTIVATION OF TRYPSIN AND CHYMOTRYPSIN. Pancreatic juice obtained from a fistula commonly shows no action on proteins. On entrance into the intestine the juice immediately becomes active. Apparently trypsin and chymotrypsin, as found in the pancreas and as secreted by that gland, exist not as such but as proenzymes called trypsinogen and chymotrypsinogen. These proenzymes have been obtained by Northrop and Kunitz in crystalline form and show protein characteristics. Trypsin appears to be normally activated by the substance *enterokinase* secreted by the intestinal mucosa. Kunitz has clearly demonstrated that enterokinase acts on trypsinogen enzymatically. Furthermore it should be noted that crystalline trypsinogen (Fig. 98) can be activated by traces of trypsin, the activation increasing in rapidity as more active trypsin is formed. In less pure trypsin preparations activation appears to be slowed up by the presence of an inhibitor which has been obtained in crystalline form and appears to be a polypeptide. Strong salt solutions may also activate trypsin.

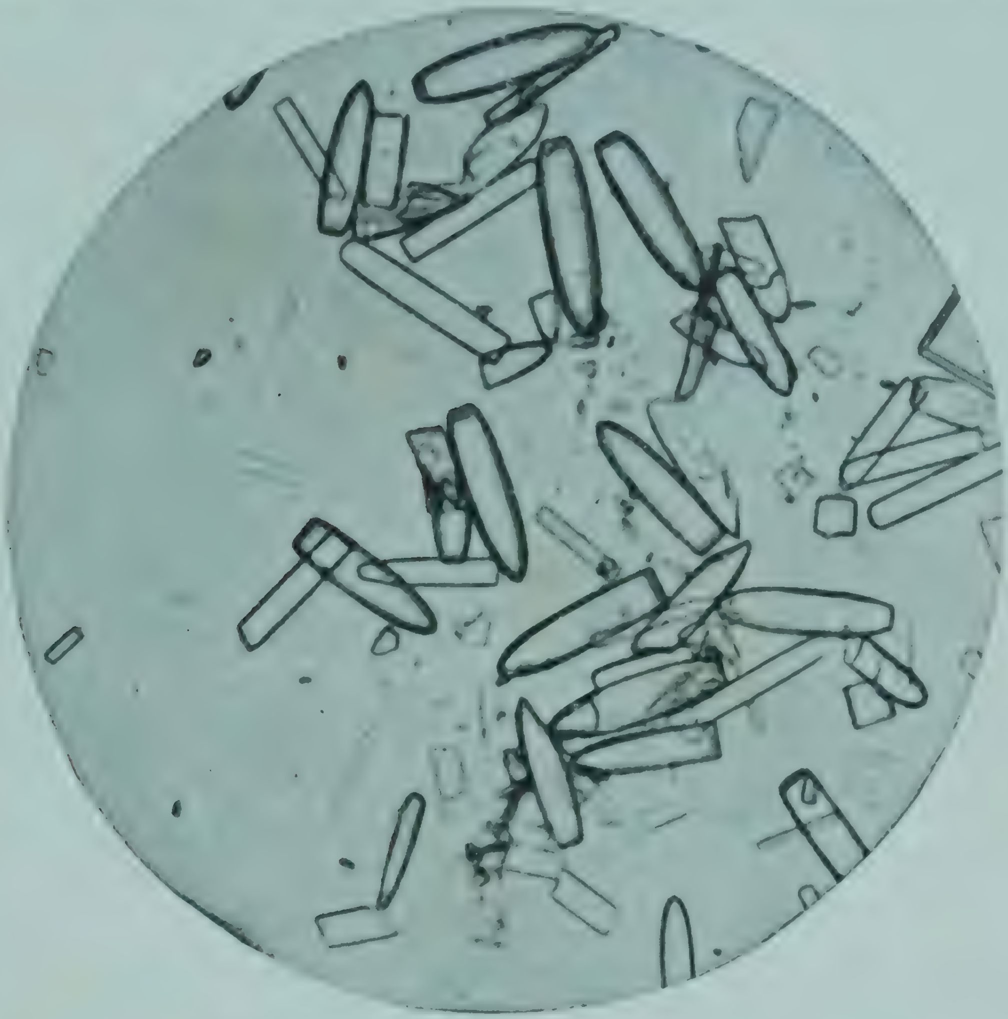

FIG. 97. CRYSTALLINE CARBOXYPOLYPEPTIDASE, PREPARED BY THE METHOD OF ANSON.
Courtesy, Dr. A. A. Plentl.

Chymotrypsinogen is apparently activated by active trypsin. Enterokinase thus does not activate chymotrypsinogen directly but indirectly through its action on trypsinogen.

Pancreatic Amylase. This is an amylolytic enzyme, or more probably a mixture of enzymes, which possesses somewhat greater digestive power than salivary amylase. As its name implies its activity is confined to the starches and similar compounds, and the products of its amylolytic action are dextrins and, ultimately, maltose.

It is probable that the saliva as a digestive fluid is not absolutely essential. The salivary amylase is destroyed by the hydrochloric acid of the gastric juice and is therefore inactive when the chyme reaches the intestine. Should undigested starch be present at this point, however, it would be quickly transformed by the active pancreatic amylase. This enzyme is not present in the pancreatic juice of infants during the first few weeks of life, indicating that a starch diet is not normal for this period.

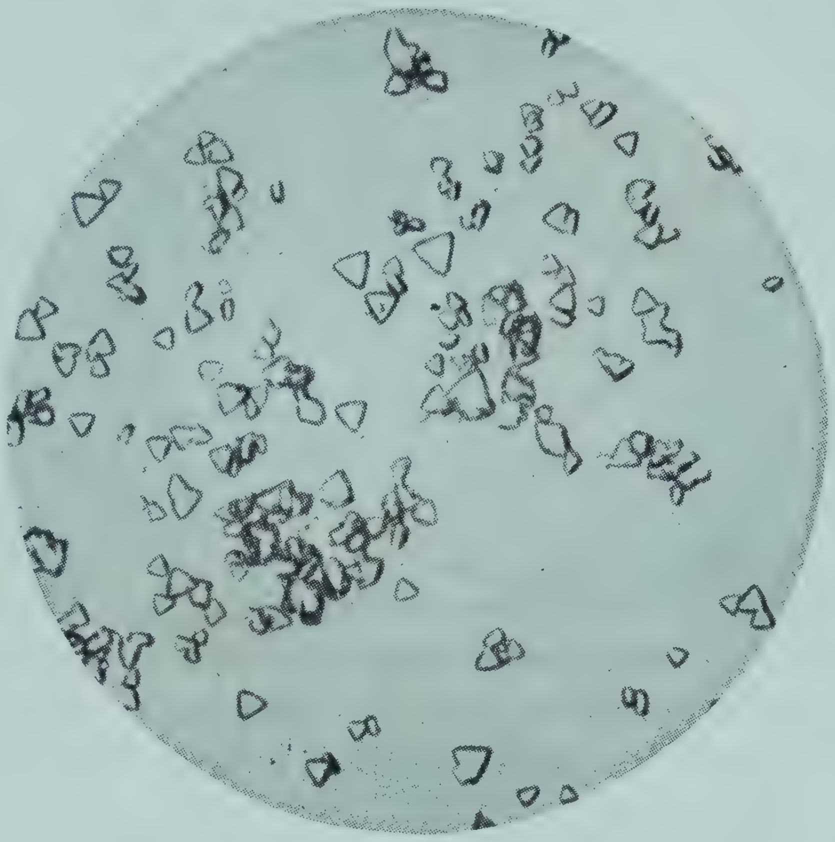

FIG. 98. TRYPSINOGEN CRYSTALS.
Courtesy, Dr. John H. Northrop.

Meyer and co-workers[4] have reported that pancreatic amylase appears to be identical with salivary amylase. They prepared crystalline amylases (see Fig. 74, p. 308) from hog pancreas, human pancreas, and human saliva, and compared them to ascertain whether enzymes of similar action from different glands of one species are more alike than corresponding enzymes from the same gland of different species. In all respects the two human amylase preparations were indistinguishable, and different from that of the hog pancreas.

Pancreatic amylase possesses protein properties. It requires ions such as Cl^- or Br^- for normal activity, and its optimal pH ranges from 6.3 to 7.2 in the presence of different neutral salts.

It has been shown that pancreatic amylase will digest raw starch. The raw starch of corn and wheat may be completely digested and absorbed by normal adults in amounts of more than 100 g. per day, whereas the raw potato starch is about 80 per cent available.

Pancreatic Lipase. This is a fat-splitting enzyme. It has the power of splitting the neutral fats of the food by hydrolysis, into fatty acid and glycerol. A typical reaction would be as follows:

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[4] Meyer, Fischer, Bernfeld, and Duckert: *Arch. Biochem.*, 18, 203 (1948).

$$\underset{\textbf{Palmitin}}{C_3H_5(OOCC_{15}H_{31})_3} + 3H_2O \rightarrow \underset{\textbf{Palmitic acid}}{3(C_{15}H_{31}COOH)} + \underset{\textbf{Glycerol}}{C_3H_5(OH)_3}$$

Pancreatic lipase is very unstable and is easily rendered inert by the action of acid. For this reason it is not possible to prepare an extract having a satisfactory fat-splitting power from a pancreas which has been removed from the organism for a sufficiently long time to have become acid in reaction.

Fodor[5] has reported that, in addition to lipase, hog pancreas contains a monovalent alcohol esterase. Although the different activities have not as yet been localized in separate protein fractions, the triglyceride-splitting activity is generally more resistant to heat, alkali, and trypsin than is the esterase activity.

Pancreatic lipase is undoubtedly the most important fat-splitting enzyme in the digestive tract. In the absence of pancreatic lipase, for example, when the pancreatic duct is obstructed by disease, the fat of the diet appears in the undigested form in the feces; this condition is known as steatorrhea. The enzyme is water-soluble and presumably a protein, although sufficiently pure preparations to prove this have not as yet been obtained, nor has the enzyme been crystallized. Its optimum pH is about 7. The action of the enzyme on fats is obviously dependent in large measure upon the surface of fat available; thus the more highly emulsified the fat is, the more surface is exposed to the aqueous phase containing the enzyme, and the more rapid is lipase action. Emulsification of fats in the digestive tract is facilitated by the presence of *bile*, because of the lowering of surface tension brought about by the bile salts (see Chapter 18). The bile also aids in fat digestion by facilitating removal of the end products of lipase action. It is believed by some that the bile actually activates pancreatic lipase; it appears more probable that the acceleration in lipase activity in the presence of bile is due to physiocochemical action by the bile salts in facilitating closer contact between the water-soluble lipase and the fat globule.

PREPARATION OF AN ARTIFICIAL PANCREATIC JUICE

After removing the fat from the pancreas of a pig or sheep, finely divide the organ by means of scissors and grind it in a mortar. If convenient, the use of an ordinary meat chopper is a very satisfactory means of preparing the pancreas.

When finely divided as above, the pancreas should be placed in a 500-ml. flask, about 150 ml. of 30 per cent alcohol added, and the flask and contents shaken frequently for 24 hours. (What is the reaction of this alcoholic extract at the end of this period, and why?) Strain the alcoholic extract through cheesecloth, filter, nearly neutralize with potassium hydroxide solution, and then exactly neutralize it to litmus with 0.5 per cent sodium carbonate.

PRODUCTS OF PANCREATIC DIGESTION OF PROTEIN

Into a 250-ml. flask introduce 20 g. of casein, 10 ml. of the artificial pancreatic juice prepared as described above, and 100 ml. of 1 per cent sodium carbonate. Allow to digest at 40° C. for 8 to 10 days with the addition of a few milliliters each of chloroform and toluene, the flask being stoppered with

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[5] Fodor: *Arch. Biochem.*, **26**, 307 (1950).

cotton. As the chloroform and toluene evaporate they must be renewed. Heat the mixture to boiling, and at the boiling point add acetic acid drop by drop until the mixture is acid in reaction. Cool and filter.

To 5 ml. of the filtrate add bromine water drop by drop. Note the development of a pink color which disappears in the presence of an excess of the reagent. This reaction indicates the presence of tryptophan.

To another 5-ml. portion of the filtrate add 10 drops of concentrated sulfuric acid and 10 ml. of a 10 per cent solution of mercuric sulfate in 5 per cent sulfuric acid. After mixing and allowing to stand for a few minutes, filter off the yellow precipitate which forms. This is an impure mercury compound of tryptophan.[6] Filter off the precipitate, reserving the filtrate, and wash the precipitate on the filter paper thoroughly with several small portions of water.

To small portions of the precipitate apply the Hopkins-Cole, xanthoproteic, and Millon tests. Tryptophan gives a positive reaction with the first two of these tests and is responsible for the Hopkins-Cole reaction as applied to protein.

Test portions of the filtrate from the mercuric precipitate by the Hopkins-Cole, xanthoproteic, and Millon reactions. Tyrosine responds to the latter two tests.

To the remainder of the original filtrate add a few drops of ammonia[7] (enough to make it slightly alkaline) and evaporate to a volume of 10 to 20 ml., using at first a free flame and completing the evaporation on a water bath. Transfer to a beaker and allow to stand for 1 or 2 days. Examine microscopically the crystals which separate out. Tyrosine crystallizes in sheaves of needles (see Fig. 44). Leucine forms small rosettes. Apply Mörner's reaction for tyrosine (see p. 138).

GENERAL EXPERIMENTS ON PANCREATIC DIGESTION

EXPERIMENTS ON TRYPSIN[8]

1. ***The Most Favorable Reaction for Tryptic Digestion.*** Prepare five tubes as follows:

a. 3 ml. of neutral pancreatic extract + 3 ml. of water. pH 7.

b. 3 ml. of neutral pancreatic extract + 3 ml. of water + 1 drop of phenolphthalein solution + 0.5 per cent Na_2CO_3 to first faint pink color. pH 8.3.

c. Same as (*b*) but add Na_2CO_3 until the pink color no longer deepens. pH 10.

d. 3 ml. of neutral pancreatic extract + 3 ml. of 2 per cent boric acid solution. pH 5.

e. 3 ml. of neutral pancreatic extract + 3 ml. of 0.6 per cent HCl. pH 3.

Add a small piece of fibrin[9] to the contents of each tube and keep them at 40° C., noting the progress of digestion. In what reactions does trypsin act and what is the optimum pH? How do the indications of the digestion of fibrin by trypsin differ from the indications of the digestion of fibrin by pepsin? Is the same degree of swelling of the protein noted?

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[6] It has been claimed that a similar yellow precipitate forms in the presence of tyrosine, cystine, and polypeptides.

[7] If the solution is alkaline in reaction owing to the presence of fixed alkali while it is being concentrated, the amino acids will be broken down and ammonia will be liberated. Ammonia in slight excess does not cause such decomposition.

[8] For these experiments as well as for those on the other pancreatic enzymes commercial preparations of trypsin and pancreatin may be employed.

[9] Congo red fibrin (see Appendix) may be used in this and the following tests on tryptic digestion. If Congo red fibrin is used the experiments should be carried out at room temperature. Also in Exp. (*b*) and (*c*) phenolphthalein should not be added, but the proper amount of alkali as determined in a separate test. Buffer solutions of suitable pH may also be used in these tests instead of the acids and alkalies suggested.

2. The Most Favorable Temperature. (For this and the following series of experiments under tryptic digestion use the neutral extract plus an equal volume of 0.5 per cent sodium carbonate.) In each of four tubes place 5 ml. of alkaline pancreatic extract. Immerse one tube in ice water, keep a second at room temperature, and place a third in the incubator or water bath at 40° C. Boil the contents of the fourth for a few moments, then cool and also keep it at 40° C. Into each tube introduce a small piece of fibrin and note the progress of digestion. In which tube does the most rapid digestion occur? What is the reason?

3. Demonstration of the Action of Enterokinase on Trypsin.

a. PREPARATION OF ENTEROKINASE. Grind 5 g. of fresh duodenal mucosa of the hog with a little sand. Gradually add 50 ml. of water during the grinding process. Strain through cheesecloth.

A better preparation is made by drying the mucosa. From the upper three feet of the intestine of the hog scrape off the mucous membrane with a knife, or, better, a glass plate. Shake with three volumes of acetone. Let stand two hours. Filter. Wash the residue again with the same amount of acetone, then with a mixture of acetone and ether, and finally twice with ether. Dry in air and pulverize. A 1:50 extract of this powder in water may be used. The powder keeps indefinitely. The enterokinase may be further purified.

b. PREPARATION OF KINASE-FREE TRYPSIN SOLUTION. Immediately after killing the animal, grind hog pancreas in a meat chopper and dry with acetone and ether as in preparation of enterokinase (see above). Glycerol extracts of this dried pancreas (1:10) may be used. Kinase-free trypsin may also be further purified.

c. DEMONSTRATION OF ACTION OF ENTEROKINASE. Prepare five tubes as follows:

(1) 2 ml. of pancreas extract + 5 ml. of water.
(2) 2 ml. of pancreas extract + 1 ml. of duodenal extract + 4 ml. of water.
(3) 1 ml. of duodenal extract + 6 ml. of water.
(4) 2 ml. of pancreas extract + 1 ml. of duodenal extract + 4 ml. of water.
(5) 2 ml. of pancreas extract + 1 ml. duodenal extract (boiled) + 4 ml. of water.

Boil contents of Tube 4 for 5 minutes and cool to 40° C. Keep all tubes at 40° for 20 minutes for activation. Add 1 ml. of 0.5 per cent sodium carbonate to each tube and the same quantity (about the size of a pea) of fresh fibrin. Shake the tubes and place at 40° C. Observe frequently during the course of an hour. Tube 2 should show the most rapid digestion. Why?

4. Quantitative Determination of Tryptic Activity.[10] See Spencer's Method, p. 390.

EXPERIMENTS ON PANCREATIC AMYLASE

1. Demonstration of Presence of Amylase in the Pancreas. Into a test tube introduce 5 ml. of starch paste and 2 ml. of pancreatic extract. Shake and put in a water bath at 40° C. for 30 minutes. Divide into two parts. Test one with iodine for undigested starch and the other by Benedict's test for reduc-

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[10] A number of proteolytic enzymes may be quantitatively determined by means of the fibrin-plate method. This procedure is both convenient and sensitive. Astrup and Alkjaersig *Arch. Biochem. Bioph.*, 37, 99 (1952).

ing sugar. The reducing sugar formed by pancreatic amylase is maltose as is the case with salivary amylase.

2. *Quantitative Determination of Amylolytic Activity: Method of Willstätter, Waldschmidt-Leitz, and Hesse.* In this method the reducing sugar formed is determined by hypoiodite titration.

Into a 50-ml. cylinder (with a ground-in stopper) introduce 25 ml. of a freshly prepared 1 per cent solution of soluble starch (see the Appendix), 10 ml. of buffer solution pH 6.8 (5.1 ml. of 0.2 M KH_2PO_4 + 4.9 ml. of 0.2 M Na_2HPO_4), and 1 ml. of 0.2 N NaCl. Mix and bring to a temperature of 37° C. Add the enzyme solution to be tested. Mix and return at once to the bath. Keep at 37° C. for exactly ten minutes. Then add 2 ml. of N HCl to stop the action. Wash the contents of the cylinder with a little water into an Erlenmeyer flask. Add 0.6 ml. of 0.1 N iodine solution for each mg. of maltose expected. Then add drop by drop with shaking 0.1 N NaOH sufficient to neutralize the added HCl and to change the primary phosphate of the buffer to secondary form (for both of which 30 ml. is required) and a further amount 1.5 times the volume of the iodine solution used. Let stand 15 minutes, acidify with dilute H_2SO_4, and titrate the excess iodine with 0.1 N thiosulfate. The iodine taken up by the starch and the enzyme solution is found in a control determination. One ml. of 0.1 N I is equivalent to 17.15 mg. of $C_{12}H_{22}O_{11}$ or maltose.

CALCULATION. The equation for a monomolecular reaction is

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

Apply this formula to the determination above: a is the amount of substrate (not the full 0.25 g. of starch but 75 per cent of this, or 0.1875, as representing the practical limits of saccharification of the starch); t is the time (10 minutes). Assume the iodine required to be 2.29 ml. and the blank determination 0.53 ml. Then the iodine taken up by the maltose will be 2.29 − 0.53 = 1.76 ml. of 0.1 N I, equivalent to 0.0302 g. of maltose, and

$$k = \frac{1}{10} \log \frac{0.1875}{0.1875 - 0.0302} = 0.0076$$

This reaction constant expresses also the number of units of amylase in the amount of amylase preparation used. The unit of amylase is 100 times the amount required under the given conditions to give a constant of 0.01. This is approximately the amount in 2 cg. of dried pancreas. The amylolytic strength of a preparation may be expressed in units per cg. For plant amylase a buffer of pH 5 should be used. For accurate work the amount of enzyme used should be such as to give a constant of 0.01 − 0.03 or hydrolysis of not over 40 per cent of the substrate.

EXPERIMENTS ON PANCREATIC LIPASE[11]

1. *Influence of Bile on Action of Lipase.* Prepare five test tubes as follows:

***a.* 0.5 ml. of olive oil + 5 ml. of neutral pancreatic extract + 4.5 ml. of water.**

***b.* 0.5 ml. of olive oil + 9.5 ml. of water.**

***c.* 0.5 ml. of olive oil + 8.5 ml. of water + 1 ml. of bile.**

***d.* 0.5 ml. of olive oil + 5 ml. of neutral pancreatic extract + 3.5 ml. of water + 1 ml. of bile.**

***e.* 5 ml. of neutral pancreatic extract + 4 ml. of water + 1 ml. of bile.**

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[11] A vegetable lipase preparation may be made as described on p. 331.

Shake the tubes thoroughly, add a drop of toluene to each, and place them in an incubator or water bath at 40° C. for 24 hours. At the end of this period add a drop of phenolphthalein to each tube and titrate with 0.05 N NaOH to a permanent pink color. Shake the tube during the titration. Record the amount of 0.05 N alkali necessary to neutralize the contents of each tube. Which tube required the most? Why?

2. *Litmus-milk Test.* Into each of two test tubes introduce 10 ml. of milk and a small amount of litmus solution.[12] To the contents of one tube add 3 ml. of neutral pancreatic extract[13] and to the contents of the other tube add 3 ml. of water or of boiled neutral pancreatic extract. Keep the tubes at 40° C. and note any changes which may occur. What is the result and how do you explain it?

3. *Copper Soap Test for Lipase.* Prepare a 2:100 agar-agar solution, mix with an equal volume of 5:100 starch paste, incorporate in this mass about 1/40 of its volume of the neutral fat desired (butter, lard, etc.), heat with constant agitation until a homogeneous emulsion is produced, pour into a Petri dish, and cool rapidly. With a fine pipet distribute on the surface of the solidified mass small drops of the liquid to be tested, keep 1 hour at 38° C., pour a saturated aqueous $CuSO_4$ solution over the surface, allow to stand 10 minutes, and rinse with H_2O. The presence of lipase is shown by the appearance of beautiful bluish-green spots. These are copper soap. The addition of the starch, which is not indispensable, produces a rather white opaque background against which the spots appear very distinct.

4. *Ethyl Butyrate Test.* Into each of two test tubes introduce 4 ml. of water, 2 ml. of ethyl butyrate, $C_3H_7COO{\cdot}C_2H_5$, and a small amount of litmus powder. To the contents of one tube add 4 ml. of neutral pancreatic extract and to the contents of the other tube add 4 ml. of water or of boiled neutral pancreatic extract. Keep the tubes at 40° C. and observe any change which may occur. What is the result and how do you explain it? Write the equation for the reaction which has taken place.

5. *Quantitative Estimation of Lipase: Method of Willstätter, Waldschmidt-Leitz, and Memmen.* A constant degree of activation is obtained by the addition of albumin and calcium salts. An adsorption compound fat-Ca oleate-albumin-lipase very favorable to lipase action is believed to be formed.

Into a wide-mouth 30-ml. flask with a ground glass stopper, introduce the enzyme preparation made up to 10 ml. with water, then 2.5 g. of olive oil and 2 ml. of buffer (0.66 ml. of N NH_4OH + 1.34 ml. of N NH_4Cl) and 0.5 ml. of 2 per cent $CaCl_2$. Shake a little and add 0.5 ml. of 3 per cent albumin solution. Shake by hand uniformly and strongly for 3 minutes to get a stable characteristic emulsion. Put in a thermostat at 30° C. for 57 minutes (total time of lipase action 60 minutes). Wash into an Erlenmeyer flask with 96 per cent alcohol to make a volume of about 125 ml. Add 20 ml. of ether and mix. This stops lipase action. Add 12 drops of 1 per cent solution of thymolphthalein and titrate with 0.1 N alcoholic KOH to a distinct blue color. From the reading subtract the titration value of the buffer (13.4 ml. of 0.1 N KOH) and of the enzyme solution. A lipase unit is the amount required under the specified

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[12] Litmus-milk *powder* may be used if desired. To prepare it add 1 part of powdered litmus to 50 parts of dried milk powder. For use in testing, 1 part of powdered litmus-milk may be added to 9 parts of water.

[13] Commercial *pancreatin* may be used in this test if desired.

conditions to split 24 per cent of 2.5 g. of olive oil of a saponification value of 185 (meaning that 1 g. of the olive oil completely hydrolyzed requires for neutralization 185 mg. of KOH).

CALCULATION. One ml. of 0.1 N KOH = 5.61 mg. of KOH. If 12.5 ml. of 0.1 N KOH (corrected for control) were required 12.5 × 5.61 = 70.6 mg. of KOH. Then $\frac{70.6 \times 100}{185 \times 2.5} = 15.1$ per cent split. If the splitting is more than 24 or less than 10 per cent, repeat. Ten per cent decomposition by pancreatic lipase corresponds to a lipase value of 0.028, and 24 per cent to 1.0. Add to 0.28 an amount of 0.044 for each per cent of decomposition above 10 and up to 24. If using other than pancreatic lipase or if other conditions are altered, a special curve of variations of percentage hydrolysis with different amounts of enzyme must be plotted from experimental data obtained using the enzyme in question. Concentration of lipase may be expressed as units per cg. of substance.

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17

Intestinal Digestion

The digestive processes carried out by the enzymes of the pancreatic juice occur within the lumen of the intestine. It is probable that peptic digestion also continues in the upper part of the duodenum until the acid chyme has been neutralized by the pancreatic and intestinal juices. However, we shall consider in this chapter only those digestive processes which are effected by enzymes secreted in the intestinal juice (succus entericus) and those present in the intestinal mucosa.

The intestinal juice is most abundant in the duodenum and jejunum and is of two types: (*a*) a secretion poor in enzyme content but rich in mucin which is produced periodically at about two-hour intervals, and (*b*) a digestive juice which is secreted in response to a meal, probably through mechanical stimulation of the intrinsic nerves of the intestine. A hormone *enterocrinin*, liberated by the mucosa in response to a meal, may be concerned in stimulation of the intestinal glands.

The reaction of the intestinal contents is influenced by the state of digestion and the relative volumes of acid chyme, pancreatic and intestinal juices, and bile present in any given segment of intestine. The reaction of the small-intestine contents is frequently acid, and the pH is usually considerably lower than that of the intestinal juice. *Intestinal secretions* collected at intervals from the jejunum through the colon contain about the same concentrations of cations, but progressively higher concentrations of bicarbonate and lower concentrations of chloride. Thus the intestinal secretions are more alkaline in the lower reaches of the intestine.

The enzymes of the intestine are of great importance, since their action, in some cases, supplements that of other digestive enzymes. They also serve to complete the digestive process by catalyzing the further hydrolysis of products of other digestive reactions. The enzymes include (1) *peptidases* (aminopolypeptidases and dipeptidases); (2) *carbohydrases* (sucrase, maltase, and lactase); (3) enzymes acting on nucleic acids, including a nonspecific *phosphatase;* (4) *lecithinase*. Some of these enzymes are not actually present in the intestinal juice, or occur there in only small amounts. Examples of such enzymes are lactase, nucleotidase, and nucleosidase. These enzymes evidently exert their action at the surface of the mucosal cells or intracellularly. These latter sites of action may also be important in the case of other intestinal enzymes.

Peptidases. AMINOPOLYPEPTIDASES. The aminopolypeptidases of the intestinal mucosa act on the products of peptic and tryptic digestion to catalyze the splitting of the peptide linkage adjacent to the end of polypeptide chains bearing the free amino group. The products are a free amino acid and a polypeptide of lower molecular weight. These enzymes supplement the effect of carboxypolypeptidases of pancreatic juice which have their site of action at the carboxyl end of the polypeptide chain. Complete hydrolysis of polypeptides may be effected by aminopolypeptidases, since they slowly hydrolyze synthetic dipeptides; but this result is better accomplished by the dipeptidases. Some structural specificity is undoubtedly involved in aminopolypeptidase action; thus leucylpeptidase in hog intestinal juice is especially active in hydrolyzing peptides containing leucine as a terminal amino acid.

DIPEPTIDASES. The dipeptidases have relatively little action on polypeptides but split dipeptides rapidly.

The combined activity of aminopolypeptidases and dipeptidases was once attributed to a single enzyme called *erepsin*. This name is now used only as descriptive of the over-all results of these enzymes. *Ereptic enzymes* are found not only in intestinal mucosa but in other plant and animal tissues as well. None of these enzymes has been crystallized. Since the ereptic enzymes (and carboxypolypeptidase) act only on peptide linkages adjacent to the end of a polypeptide or peptide chain, they are referred to as *exopeptidases* to distinguish them from pepsin and the tryptic enzymes which act on centrally located peptide bonds. Through the combined action of all these enzymes, protein is finally hydrolyzed to individual amino acids.

Carbohydrases. SUCRASE. The three carbohydrases sucrase, maltase, and lactase are also important enzymes of the intestinal mucosa. The sucrase acts upon sucrose and inverts it with the formation of glucose and fructose. Sucrases may also be obtained from several vegetable sources. For investigational purposes sucrase is ordinarily obtained from yeast (see p. 331). Intestinal sucrase has an optimum pH of 5 to 7; yeast invertase acts best at pH 4.5.

LACTASE. This enzyme splits lactose with the consequent formation of glucose and galactose. Freshly prepared suspensions of intestinal mucosal tissue are much more active than water extracts, or the intestinal juice, indicating that the activity is intimately associated with the mucosal cells. The optimum pH for intestinal lactase is about 5.4 to 6.0.

MALTASE. This enzyme possesses the power of splitting maltose, the end product of the digestion of starch by amylase, into glucose. Maltase is best prepared from yeast. Its optimum pH is 6.7 to 7.2.

Nucleases. A *polynucleotidase* in intestinal juice results in the depolymerization and break up of nucleic acid to nucleotides. The nucleotides are then hydrolyzed by a *nucleotidase* (a phosphatase) to give purine and pyrimidine nucleosides and phosphoric acid. A *nucleosidase* decomposes the purine nucleosides to the purine base and ribose (or deoxyribose). The pyrimidine nucleosides are absorbed unchanged but are decomposed by enzymes found in other organs. The intestinal phosphatase responsible for the hydrolysis of the phosphoric acid ester linkage of nucleotides is nonspecific in its action, since it attacks other phosphoric acid ester substrates such as glycerophosphate and hexose phosphates. A part of the alkaline phosphatase activity of normal plasma is due to the intestinal phosphatase which has entered the blood.

Lecithinases. Enzymes which hydrolyze lecithins to various products, depending upon the experimental conditions and time of action, are present in intestinal mucosa.

Enterokinase. This substance is discussed in Chapter 16. Though present in intestinal juice, it is not a digestive enzyme but activates the tryptic enzymes.

GENERAL EXPERIMENTS ON INTESTINAL DIGESTION

Preparation of Intestinal Extract. **Wash a piece of hog intestine about 18 inches long. Run it through a meat-chopper or, better, scrape off the mucosa with a knife or piece of glass. Rub in a mortar with sand. Add 5 volumes of water and a little chloroform and let stand at room temperature for 24 hours. If necessary to expedite laboratory work the extract may be used after two hours. Strain through cloth or loose cotton. This extract may be used for the general experiments on phosphatase, ereptic enzymes, and sucrase. The presence of some mucosal cells in suspension increases the enzyme activity.**

EXPERIMENT ON INTESTINAL PHOSPHATASE

Demonstration of Action of Intestinal Phosphatase on Nucleic Acid and on Sodium Glycerophosphate. **Prepare a 2 per cent solution of yeast nucleic acid with the aid of just sufficient dilute NaOH to make the resulting solution pink to phenolphthalein (pH 8.6). Then prepare a 2 per cent solution of sodium glycerophosphate and make just pink to phenolphthalein. To each of four test tubes add 10 ml. of the intestinal extract prepared as above. Boil two—Tubes 2 and 4—for one to two minutes. To Tubes 1 and 2 then add 10 ml. of the 2 per cent nucleic acid solution and to tubes 3 and 4, 5 ml. of the glycerophosphate solution. Add 2 to 3 ml. each of toluene and chloroform to each mixture. Keep at 38° C. for 24 hours.**

Heat the tubes to boiling in a water bath to coagulate protein. Add 5 ml. of 5 per cent HCl and allow to stand for one hour. This precipitates any unchanged nucleic acid. Filter and take aliquots of the filtrate (about 10 ml.). Precipitate the phosphate from each mixture by adding 5 ml. of magnesia mixture and 5 ml. of ammonia. Allow to stand over night. A heavy precipitate of magnesium ammonium phosphate should be found in Tubes 1 and 3, indicating that the phosphoric acid of the nucleic acid and of the glycerophosphate has been liberated by the phosphatase of the intestinal extract. The control should show only a slight precipitate.

EXPERIMENTS ON PEPTIDASES

1. Demonstration of Peptidase Activity. **To about 5 ml. of a 1 per cent solution of peptone in a test tube add about 10 ml. of the intestinal extract prepared as described above. Prepare a second tube containing a like amount of peptone solution, but boil the intestinal extract before introducing it. Place the two tubes at 38° C. for two to three days. At the end of that period heat the contents of each tube to boiling, filter, and try the biuret test on each filtrate. In making these tests, care should be taken to use like amounts of filtrate, sodium hydroxide, and copper sulfate in each test in order that the drawing of correct conclusions may be facilitated. The contents of the tube which contained the boiled extract should show a deep pink color with the biuret test, owing to the peptone still present. On the other hand, the biuret**

test upon the contents of the tube containing the unboiled extract should be negative or exhibit, at the most, a faint pink or blue color, signifying that the peptone, through the influence of the intestinal extract, has been transformed, in great part at least, into simple peptides and amino acids which do not respond to the biuret test. To other portions of the filtrates add a few drops of bromine water. A violet color indicates free tryptophan and hence that amino acids have been liberated. Proteinase in the extract changes the peptone to polypeptides. The latter are broken down by aminopolypeptidase to simple peptides which under the action of dipeptidase yield amino acids. The action of several enzymes is thus involved.

2. Demonstration of Dipeptidase Using Glycyltryptophan. **Introduce 5 ml. of glycyltryptophan solution into each of two tubes. To one add 5 ml. of intestinal extract, to the other 5 ml. of boiled extract. Let stand over night. Add a few drops of bromine water to each. A violet color indicates free tryptophan and hence the presence of dipeptidase.**

3. Quantitative Determination of Dipeptidase. **To 25 ml. of 0.05 N glycylglycine add NaOH to pH 8. Warm to 37° C. Add enzyme solution. Carry out a formol titration on an aliquot at once (control) and repeat at intervals. Choose a determination representing less than 30 per cent digestion. Calculate k in: $k = (1/t) \cdot \log a/(a - x)$. Activity equals k/g. enzyme preparation.**

EXPERIMENTS ON CARBOHYDRASES

1. Demonstration of Sucrase. **To about 5 ml. of a 1 per cent solution of sucrose, in a test tube, add about 1 ml. of intestinal extract, prepared as described above, and a few drops of chloroform. Prepare a control tube in which the intestinal extract is boiled before being added to the sugar solution. Let stand for 24 hours. Heat the mixture to boiling to coagulate the protein material, filter, and test the filtrate by Benedict's test (see p. 66). The tube containing the boiled extract should give no response to Benedict's test, whereas the tube containing the unboiled extract should reduce the Benedict's solution. This reduction is due to the formation of glucose and fructose from the sucrose through the action of the enzyme sucrase which is present in the intestinal epithelium.**

For the preparation of vegetable sucrase see p. 331.

2. Demonstration of Lactase and Maltase. **Repeat the above experiment but use 1 per cent solutions of lactose and maltose. Is there any evidence of lactase and maltase activity?**

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18

Bile and Liver Function

Secretion of Bile. The bile is secreted continuously by the liver and passes into the intestine through the common bile duct which opens near the pylorus. This process is continuous even during prolonged fasting, provided there is no obstruction. The ingestion of food, however, increases the secretion. Meat is effective in this respect, fat less so, and starch and sugar appear to be without effect.

Introduction of acid into the duodenum stimulates bile formation, suggesting that secretin is responsible. However, injection of secretin preparations, though leading to a copious secretion of pancreatic juice in one to three minutes, produces a much slighter secretion of bile and then only after seven to nine minutes. Ligation of the pancreatic vein prevents this action on the liver; hence it is suggested that some metabolic product of the pancreas is responsible.

Bile salts absorbed from the intestine have a marked power to stimulate bile formation. The passage of bile directly from the liver or through the emptying of the gallbladder serves to induce a further secretion. Dehydrocholic acid is an especially effective cholagogue.

Mechanism of Emptying of the Gallbladder. There appear to be at least two mechanisms active in gallbladder emptying. One of these involves the contraction of the gallbladder and the other the tone of the sphincter of Oddi at the entrance of the common bile duct into the intestine. Cream or egg yolk causes an emptying of the gallbladder apparently by inducing an active contraction of this organ, probably accompanied by a relaxation of the sphincter at the same time. The active agent is the free fatty acid liberated on digestion of these foods. The organic acids of fruits and the gastric acid have the same effect. In cases of acute cholecystitis, fats, acid fruits, and meats, which stimulate acid secretion in the stomach, should therefore be reduced in the diet in favor of cereal foods. The contraction is apparently brought about through liberation from the intestinal mucosa of a hormone, cholecystokinin, whose chemical nature is not yet determined. Magnesium sulfate promotes evacuation by causing a dilation of the sphincter.

Functions of the Bile. We may look upon the bile as an excretion as well as a secretion. In the fulfillment of its excretory function it passes such substances as lecithin, metallic compounds, cholesterol, and the decomposition products of hemoglobin into the intestine and in this way aids in removing them from the organism. As a secretion, the bile assists materially in the digestion and absorption of fats from the intestine by its emulsifying action on the fats of the diet and by facilitating the absorption of the fatty acids formed by the action of the pancreatic juice. A decreased appetite for fats has been shown in rats after the ligation of the common bile duct. A further important function of the bile is to aid in the absorption of vitamin K. Symptoms of vitamin K deficiency frequently accompany the absence of an active secretion of bile into the intestine.

Composition of Bile. The bile is a ropy, viscid fluid which is alkaline in reaction (pH 7.8) when it issues from the liver, and ordinarily possesses a decidedly bitter taste. It varies in color in the different animals, the principal variation being yellow, brown, and green. Fresh human bile from the living organism ordinarily has a yellow-brown or golden-yellow color. Post-mortem bile is variable in color. It is very difficult to determine accurately the amount of normal bile secreted during any given period. For an adult man it has been variously estimated at from 500 ml. to 1100 ml. for 24 hours. The specific gravity of the bile varies between 1.010 and 1.040, and the freezing point is about −0.56° C. As secreted by the liver, the bile is a clear, limpid fluid which contains a relatively low content of solid matter. This secretion has a specific gravity of approximately 1.010. After it reaches the gallbladder, however, it becomes mixed with mucous material from the walls of the gallbladder, and this process coupled with the continuous absorption of water and certain other components from the bile has a tendency to concentrate the secretion. Therefore the bile as we find it in the gallbladder ordinarily possesses a higher specific gravity than that of the freshly secreted fluid. The specific gravity under these conditions may run as high as 1.040. There is a decrease in inorganic salts owing to absorption, while the concentration increases the content of organic substances. Even though it is concentrated in the gallbladder, the bile remains practically isotonic with the blood because the increased content of high-molecular-weight bile salt ions is accompanied by a decrease in chloride and bicarbonate ions. The pH of bladder bile may fall as low as 6, as compared to the definitely alkaline reaction of fistula bile.

SELECTED ANALYSES ILLUSTRATING THE COMPOSITION
OF HUMAN BILE
(PARTS PER 1,000)

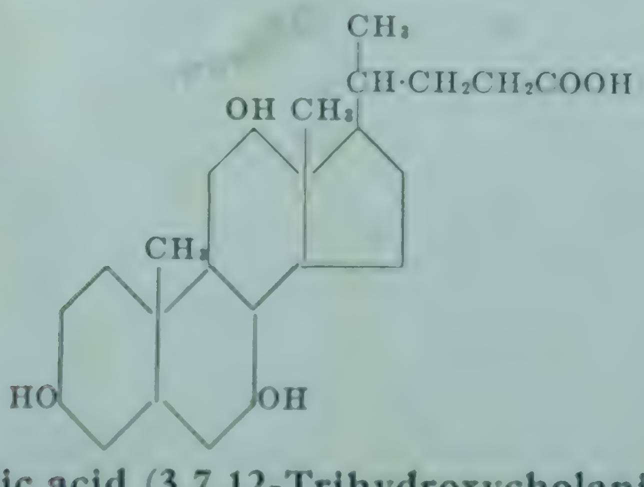
Constituent	*Fistula Bile*	*Bladder Bile*
Water	976.0	860.0
Solids	24.0	140.0
Bile acids	5.7	53.7
Mucin and pigments	8.0	41.4
Total lipides	2.9	18.8
Fatty acids	0.8	8.5
Neutral fat	0.8	1.5
Cholesterol	0.8	5.7
Phosphatides	0.5	2.2
Inorganic matter	7.4	8.5

The principal organic constituents of the bile are the salts of the bile acids, bile pigments, neutral fats, lecithin, phosphatides, nucleoprotein, mucin, and cholesterol. Inorganic constituents include chiefly sodium, chloride, and bicarbonate, some potassium, calcium, magnesium, and sulfate, and a trace of phosphate. The metals iron, copper, and zinc are also frequently present in detectable amounts. The bicarbonate content of hepatic bile is higher than that of serum; the chloride content of bile is lower than that of serum, the chloride ion being replaced largely by the organic bile salt ions.

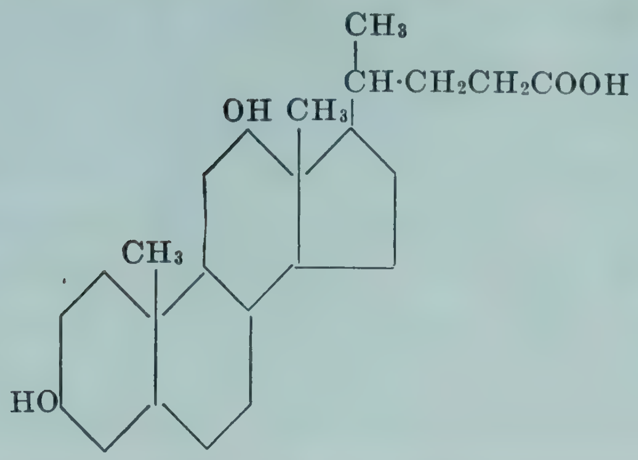
The quantitative composition of bile varies according to the source of the bile, i.e., whether the bile for analysis is obtained from the gallbladder or by means of a fistula before it reaches the gallbladder. The difference in the composition of these two types of bile is shown in the table of selected analyses on p. 409.

Bile Acids. The bile acids are elaborated exclusively as far as is known by the hepatic cells of vertebrates. They may be divided into two groups: (1) the glycocholic acid group and (2) the taurocholic acid group. In human bile glycocholic acids predominate, while taurocholic acids are more abundant in the bile of carnivora. The glycocholic acids are combinations through peptide linkage of bile acids and *glycine*, $NH_2 \cdot CH_2 \cdot COOH$. The taurocholic acids are similar combinations of bile acids with *taurine*, $NH_2 \cdot CH_2 \cdot CH_2 \cdot SO_3H$. Taurine is evidently derived in the body from cysteine.

There are several varieties of bile acids, and therefore there are several forms of glycocholic and of taurocholic acids, depending upon the nature of the bile acid entering into the combination. The principal bile acids are (1) cholic acid, $C_{24}H_{40}O_5$, with three hydroxyl groups; (2) deoxycholic acid, $C_{24}H_{40}O_4$, with two hydroxyl groups; (3) anthropodeoxycholic acid and (4) hyodeoxycholic acid which are isomeric with deoxycholic acid, differing only in the position of one hydroxyl group; and (5) lithocholic acid, $C_{24}H_{40}O_3$, which has a single hydroxyl group. Human bile contains the first three of these in the proportion of about three parts of cholic acid to one part of deoxycholic and some anthropodeoxycholic acid. Ox bile contains about 6 parts per 100 of cholic acid and about one-eighth as much deoxycholic acid. Hyodeoxycholic acid is found in hog bile and chenodeoxycholic acid (identical with anthropodeoxycholic acid) in the bile of the goose and chicken. The cholic acids are closely related in structure to cholesterol and are probably formed in the body from cholesterol or one of its immediate metabolic precursors. See the structural formulas for cholic and deoxycholic acids.



Cholic acid (3,7,12-Trihydroxycholanic acid)



Deoxycholic acid (3,12-Dihydroxycholanic acid)

The bile acids are present in the bile largely as sodium salts. The sodium glycocholate and taurocholate may be isolated in crystalline form, either as balls or rosettes of fine needles or in the form of prisms having ordinarily four or six sides (Fig. 99). The bile salts are readily soluble in water. The free acids are slightly soluble in water but readily soluble in alcohol.

The bile acids have the property of combining with fatty acids to form compounds which have been called choleic acids. These are coordination compounds, the molecules being joined through secondary valences. Fatty acids containing 16 or more carbon atoms combine with 8 molecules of bile acid; the lower fatty acids, with from 6 to 1 molecules. These compounds are soluble and diffusible in alkaline or slightly acid solution. For this reason, and because they markedly lower the surface tension in solution and thus promote emulsification, the bile acids greatly assist in the digestion and absorption of fat in the intestines. Through similar combinations they likewise assist in the absorption of cholesterol, fat-soluble vitamins, carotene, and other substances. They are also responsible for holding the cholesterol of the bile in solution.

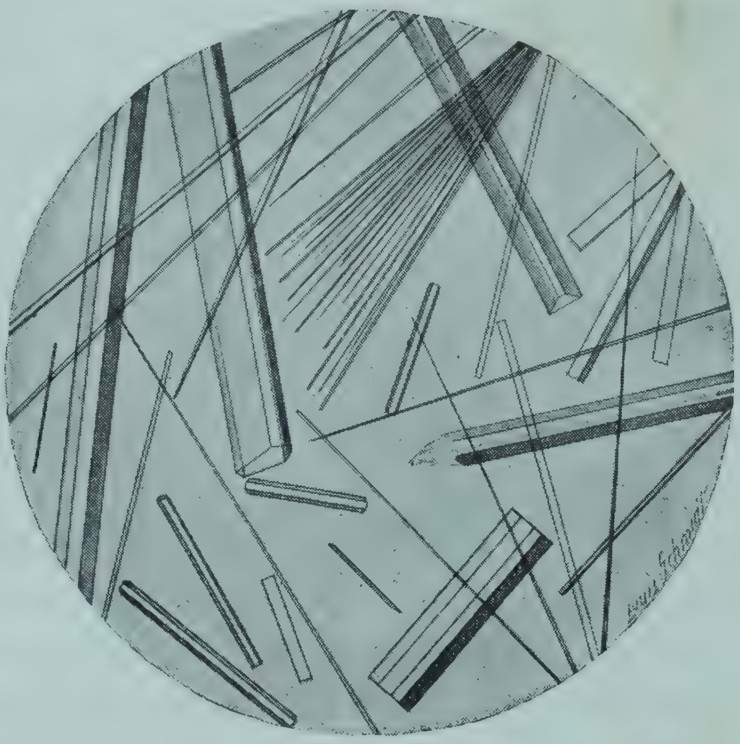


FIG. 99. BILE SALTS.

There is considerable evidence that the bile acids are used over and over again by the body. After secretion into the intestine, that fraction of the bile salts which is reabsorbed as choleic acid complexes is liberated in the intestinal mucosa. The bile salts set free, as well as any which have been absorbed as such, are carried to the liver in the portal circulation, where they become available for resecretion in the bile. This has been called the enterohepatic circulation of the bile salts. It is presumably responsible in part for the marked stimulatory (choleretic) power of the bile salts themselves over bile flow from the liver, since when the bile salts reach the liver they provide that organ with a readily available supply of a major constituent of the bile itself.

Bile Pigments. The pigments of normal bile are bilirubin and biliverdin (dehydrobilirubin). The green color predominating in certain biles as ox bile is due to biliverdin. Other types of bile like human bile commonly show the yellowish-brown color of bilirubin (Fig. 100). Modifications of these pigments may be found in gallstones or in altered bile.

The bile pigments result mainly from the breakdown of the hemoglobin of the red cells; a consideration of the structures of the bile pigments (p. 413) indicates that they are derived specifically from the heme portion of hemoglobin (see Chapter 22). Other heme derivatives in the body—such as catalase, the cytochromes, etc.—may contribute to a certain extent to bile pigment formation. The formation of bile pigments has been shown to be a property of the cells of the reticuloendothelial system. Since these include the Kupffer cells lining the intralobular capillaries of the liver, a part of the bile pigments is produced in the liver. Other important sources are the spleen, lymph nodes, and bone marrow. Wherever extravasation of blood occurs, e.g., following bruises, a conversion of blood hemoglobin to bile pigment slowly takes place. This conversion is apparently going on even in normal red cells, since small amounts of bile pigment have been isolated from this source.

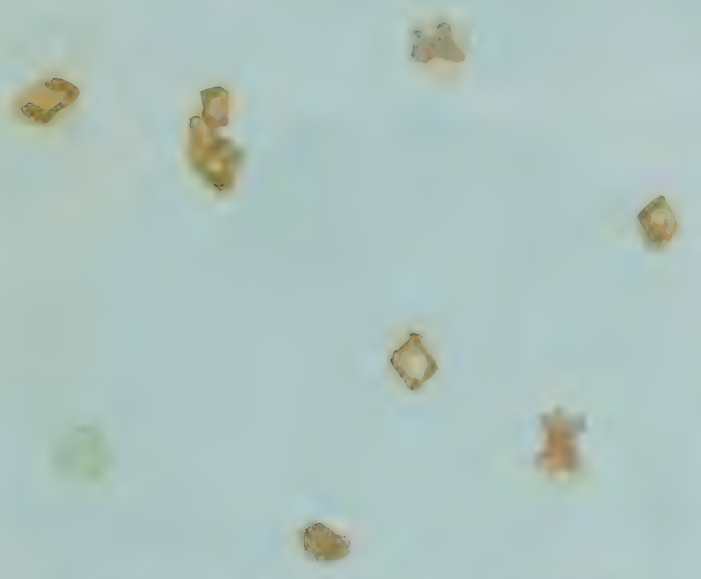
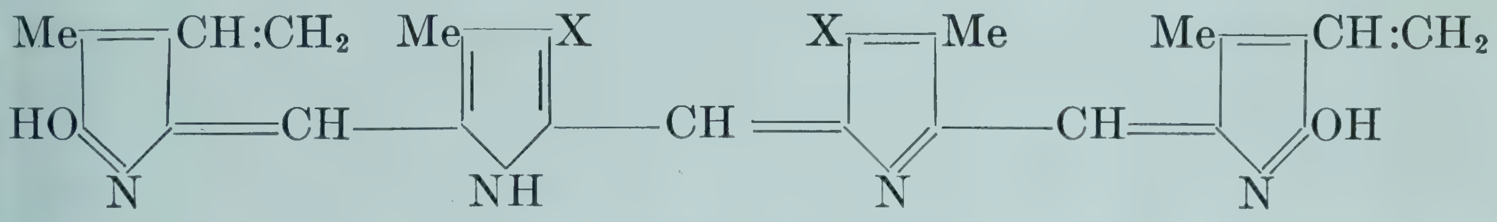


Fig. 100. Bilirubin. (Ogden.)

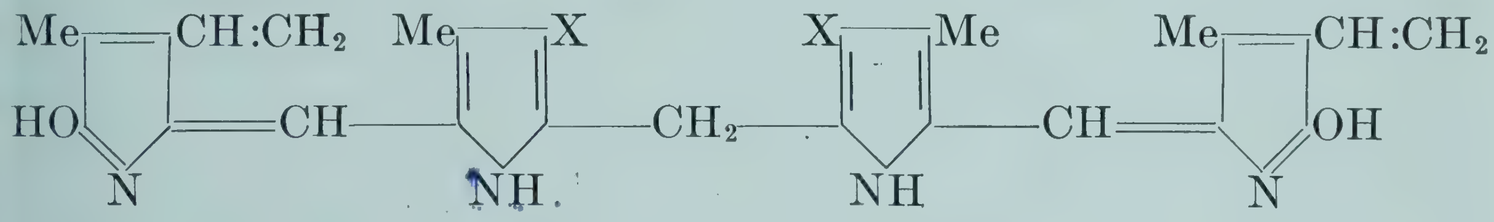
The first step in the formation of bile pigments appears to involve an oxidative scission of the porphyrin ring, to produce carbon dioxide and an open-ring compound. In this process the iron is not necessarily lost from the molecule, nor does the open-ring compound lose its affinity for globin. One such compound of globin and an open-ring iron porphyrin derivative is called choleglobin, by analogy to hemoglobin; and other compounds analogous to those found in the field of heme chemistry are known. In general, these pigments are green in color (hence the names verdohemin, verdohemoglobin, verdohemochromogen, etc.) and the opening of the porphyrin ring has apparently rendered the iron labile, so that it is easily split off by such means as treatment with dilute acid. Further steps in bile-pigment formation are obscure; apparently the iron and the globin become detached in some way to produce biliverdin, which may then be reduced to form bilirubin.

Further stages in the transformation of bile pigments involve the action of intestinal bacteria. In the intestinal lumen, bilirubin undergoes reduction by bacterial action to form the substance mesobilirubinogen. This compound ordinarily is further reduced to form stercobilinogen, which on oxidation becomes converted into stercobilin, the principal pigment of normal feces. Stercobilin has been produced in the laboratory from mesobilirubinogen by incubation of the latter with normal feces, or with bile-free feces plus added bile. A portion of the stercobilinogen and stercobilin is apparently absorbed from the intestinal tract and re-excreted by the liver; thus some gets into the blood stream and ultimately is excreted by way of the kidneys into the urine. In the urine these two pigments are known respectively as urobilinogen and urobilin. Considerable confusion has resulted from the nomenclature of these compounds; at the present time it appears to be largely a question of different names for the same substances. Normally the amounts of urobilinogen and urobilin in urine are rather small and obviously depend upon such factors as the rate of pigment production and reabsorption, and upon the efficiency of the liver in excreting the reabsorbed material. Thus the urobilinogen content of the urine will be low in simple anemias, but high in diseases associated with extensive blood destruction and where liver function is impaired.

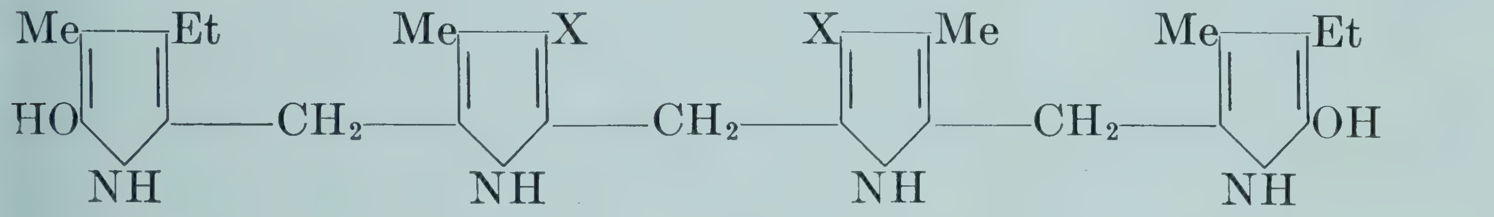
The chemical relationship between certain of the various bile pigments and their derivatives is illustrated by the following structures: (Me = CH_3; Et = C_2H_5; X = $CH_2 \cdot CH_2 \cdot COOH$).



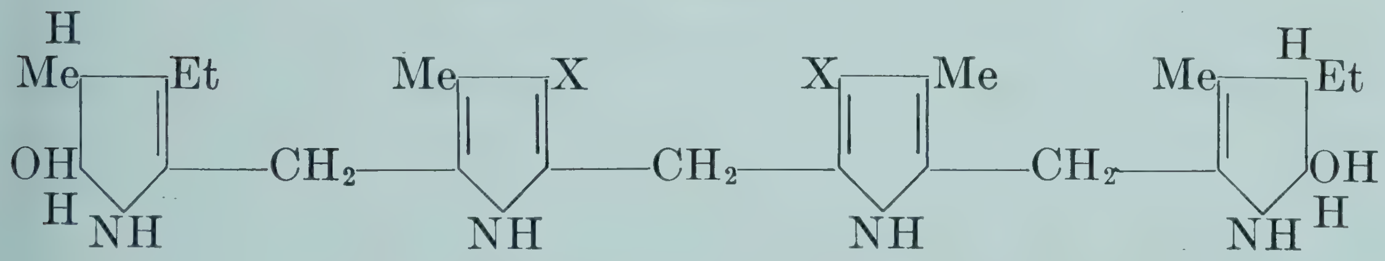
Biliverdin ($C_{33}H_{34}O_6N_4$)



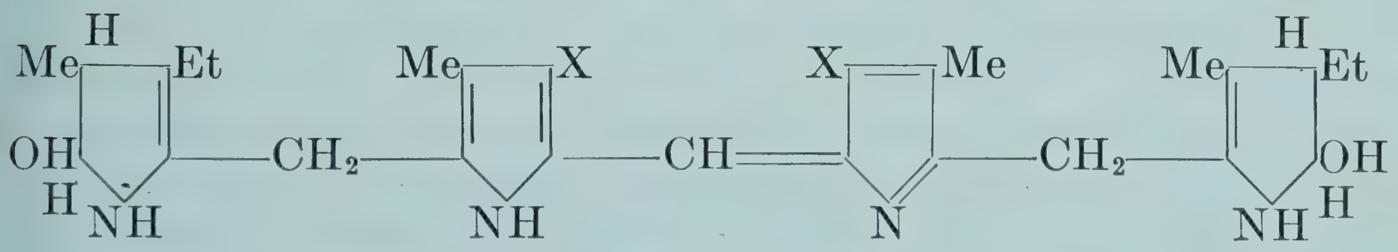
Bilirubin ($C_{33}H_{36}O_6N_4$)



Mesobilirubinogen ($C_{33}H_{44}O_6N_4$)



Stercobilinogen (**Urobilinogen,** $C_{33}H_{48}O_6N_4$)



Stercobilin (**Urobilin,** $C_{33}H_{46}O_6N_4$)

Bilirubin is insoluble in water but its alkali salts are soluble. It is soluble in chloroform and somewhat in alcohol, but very slightly in ether or benzene. Biliverdin is insoluble in water, ether, or chloroform, but soluble in alcohol. The calcium salts of the pigments are insoluble in water. Solutions of bilirubin exhibit specific light absorption only at the extreme blue end of the spectrum where the band merges into the end of the visible spectrum and is not ordinarily detectable as a discrete band in the spectroscope. If an ammoniacal solution of bilirubin-alkali in water is treated with a solution of zinc chloride, however, it shows an absorption spectrum similar to that of bilicyanin. When bilirubin is treated with diazotized sulfanilic acid, this reagent reacts with bilirubin to form a deeply colored azo dye, known as azobilirubin or azorubin. This reaction is the basis for the Van den Bergh test for bile pigments and is also used in the quantitative determination of bilirubin in biological fluids (see Chapter 23).

Biliary Calculi (Biliary Concretions, Gallstones). Biliary calculi, otherwise designated as biliary concretions or gallstones, are *frequently* formed in the gallbladder. These deposits may be divided into five classes, (1) cholesterol calculi, (2) cholesterol-calcium calculi, (3) cholesterol-calcium-pigment calculi, (4) calcium-pigment calculi, and (5) calculi made up almost entirely of inorganic material. This last class of calculus is formed principally of the carbonate and phosphate of calcium and is rarely found in man although quite common in cattle. The calcium-pigment calculus is also found in cattle, but is almost as rare in man as the inorganic calculus. This calcium-pigment calculus ordinarily consists principally of bilirubin in combination with calcium; biliverdin is sometimes present in small amount. The cholesterol calculus is the one found most frequently in man. Such calculi may be formed almost entirely of cholesterol, in which event their color is very light; or they may contain more or less pigment and inorganic matter mixed with the cholesterol, which tend to give calculi of various colors.

Our knowledge of the origin of gallstones is imperfect. Among the factors concerned may be stagnation of bile, disturbances of metabolism, and infection. Cholesterol is held in solution in bile by means of bile acids. If cholesterol excretion is increased or bile salt excretion diminished, cholesterol stones may form. Prolonged stagnation of bile may work in the same direction if the bile salt concentration is reduced by their resorption from the bladder. The resorption of alkaline bile salts tends also to decrease the pH of the bile and thus to reduce the solvent action of the bile salts on cholesterol, which is greater in alkaline than in acid solutions. Infection, with or without stagnation, provides abundant nuclei for stone formation, and chemical alterations in the bile favor the formation of cholesterol-calcium-pigment stones. The impairment in the concentrating power of the mucosa due to inflammation, through failure to keep the bile salt concentration sufficiently high, may also be a factor.

Okey[1] reports the finding of gallstones in guinea pigs being used for the study of effects of high cholesterol diets. The stones occurred only when cholesterol and riboflavin were added to diets containing 25 per cent protein, and were not found when the riboflavin concentration was decreased.

For a discussion of cholesterol see Chapter 11, Nervous Tissue.

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[1] Okey: *Proc. Soc. Exptl. Biol. Med.*, **51**, 349 (1942).

EXPERIMENTS ON BILE[2]

1. *Reaction.* Test the reaction of fresh ox bile, using suitable indicator paper. What is the approximate pH of bile?

2. *Nucleoprotein and Bile Acids.* Acidify 5 ml. of bile with acetic acid, drop by drop. Note the formation of a precipitate of nucleoprotein and bile acids.

3. *Inorganic Constituents.* Evaporate 10 ml. of bile to dryness in an evaporating dish. Fuse the residue with an excess of sodium carbonate–potassium nitrate "fusion mixture."[3] Cool, extract with 10 ml. of water, and add sufficient concentrated nitric acid to make the extract slightly acid. Filter, and test the filtrate for chloride, sulfate, and phosphate (see Chapter 28).

4. *Preparation of Bilirubin from Bile.* 500 ml. of bile from surgical drainage patients or 50 ml. of post-mortem gallbladder bile (diluted two to three times) is allowed to stand for a few hours in the refrigerator, and the supernatant fluid decanted. This is diluted several times with water and 5 per cent barium chloride solution is added with stirring. If the precipitate of barium bilirubinate does not flocculate immediately, add a few drops of 10 per cent NaOH. When the precipitate settles, the supernatant fluid is siphoned off. The precipitate is poured on a filter, washed with water on the paper, dried, and pulverized in a mortar. The powder is extracted with warm alcohol, followed by ether and chloroform, and again air-dried. The precipitate is transferred to a 50-ml. centrifuge tube, moistened with 10 per cent sulfuric acid, and washed three times in a little absolute alcohol, centrifuging and pouring off the alcohol each time. The residue is twice treated in a flask with boiling chloroform and filtered, and the chloroform evaporated, avoiding overheating toward the end. The bilirubin so obtained is rubbed into glacial acetic acid and centrifuged, the acid drained off, and the procedure repeated; the residue is air-dried. It is redissolved in boiling chloroform, filtered, and evaporated to dryness. The final product (40 to 100 mg.) is brick red in color, free from ash, and quite stable.

5. *Tests for Bile Pigments.* Practically all of these tests for bile pigments are based on the oxidation of the pigment, by a variety of reagents, with the formation of colored derivatives, e.g., mesobilirubin (yellow), mesobiliverdin (green to blue), and mesobilicyanin (blue to violet).

a. GMELIN'S TEST. To about 5 ml. of concentrated nitric acid in a test tube, carefully add 2 to 3 ml. of diluted bile so that the two fluids do not mix. At the point of contact note the various colored rings: green, blue, violet, red, and reddish-yellow. Repeat this test with different dilutions of bile and observe its delicacy.

b. ROSENBACH'S MODIFICATION OF GMELIN'S TEST. Filter 5 ml. of diluted bile through a small filter paper. Introduce a drop of concentrated nitric acid into the cone of the paper and note the succession of colors as given in Gmelin's test.

c. VAN DEN BERGH TEST. To 5 ml. of diluted bile, add 2 ml. of freshly prepared Ehrlich's diazo reagent.[4] Compare with a control on water alone. In this reaction an azo dye is formed by a coupling reaction of bilirubin with diazotized sulfanilic acid of the Ehrlich reagent. This reaction is the basis for qualitative and quantitative procedures for estimation of bile pigments in blood serum. A very sensitive test for bile pigment in urine is based on its coupling with *p*-nitrobenzene diazonium *p*-toluene sulfonate.

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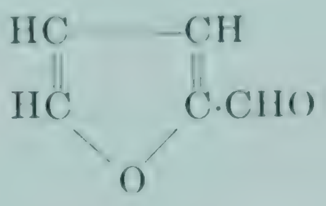
[2] For experiments on the function of bile salts which may be carried out in this connection see Chapter 19, Intestinal Absorption.

[3] See Appendix.

[4] See Appendix.

6. Tests for Bile Acids.

a. FURFURAL-H_2SO_4 TEST (MYLIUS'S MODIFICATION OF PETTENKOFER'S TEST). To approximately 5 ml. of diluted bile in a test tube add 3 drops of a very dilute (1:1000) aqueous solution of furfural:



Now run about 2 to 3 ml. of concentrated sulfuric acid carefully down the side of the tube and note the red ring at the point of contact. Upon shaking the tube the whole solution is colored red. Keep the temperature of the solution below 70° C. by cooling in running water during the mixing.

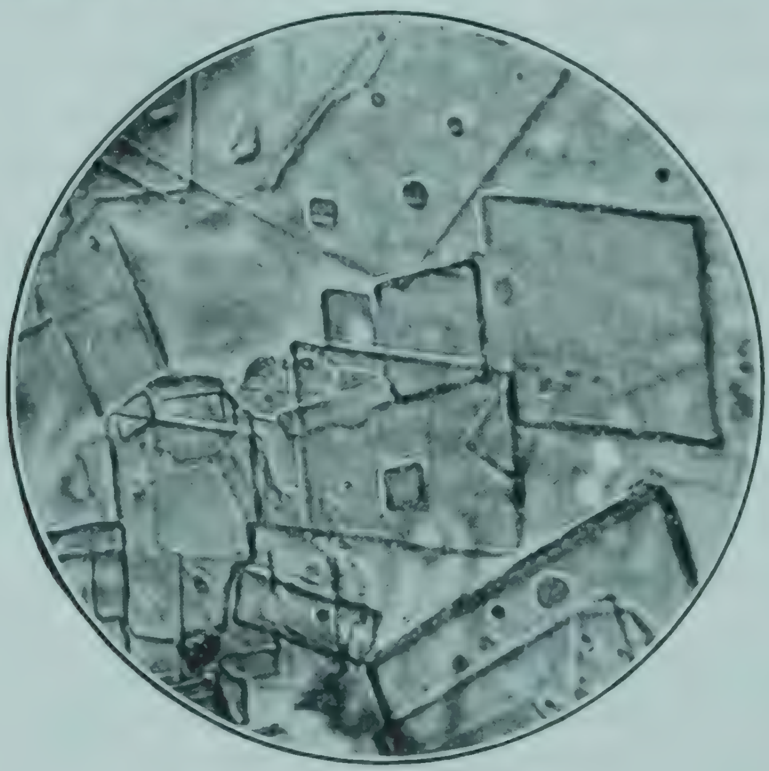


FIG. 101. CHOLESTEROL.
Courtesy, Dr. C. A. Bachhuber.

b. FOAM TEST (VON UDRÁNSZKY). To 5 ml. of diluted bile in a test tube add 3 to 4 drops of a very dilute (1:1000) aqueous solution of furfural. Place the thumb over the top of the tube and shake the tube until a thick foam is formed. By means of a small pipet add 2 to 3 drops of concentrated sulfuric acid to the foam and note the dark-pink coloration produced.

c. GREGORY AND PASCOE TEST. To 1 ml. of dilute bile solution add 6 ml. of 45 per cent H_2SO_4 and 1 ml. of 0.3 per cent furfural solution. Stopper loosely and put in a water bath at 65° C. for 30 minutes. The presence of cholic acid is indicated by a blue color in the solution. This test may be used as a quantitative method.

d. SURFACE TENSION TEST (HAY). This test is based upon the property that bile acids have of lowering the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 ml. of diluted bile in a test tube to 17° C. or lower and sprinkle a little finely pulverized sulfur upon the surface of the fluid. The presence of bile acids is indicated if the sulfur sinks to the bottom of the liquid. Prove this point by repeating the test with water instead of bile. Depending on the grade of sulfur used, this test indicates the lowering of the surface tension from 72 dynes/cm. for distilled water to values below 52–54 dynes/cm.

7. *Crystallization of Bile Salts.* To 25 ml. of undiluted bile in an evaporating dish add enough animal charcoal to form a paste, and evaporate to dryness on a water bath. Remove the residue, grind it in a mortar, and transfer it to a small flask. Add about 50 ml. of absolute alcohol and boil on a water bath for 20 minutes. Filter, and add ether to the filtrate until there is a slight permanent cloudiness. Cover the vessel and set it aside until crystallization is complete. Examine the crystals under the microscope and compare them with those shown in Fig. 99. Try one of the tests for bile acids upon some of the crystals.

8. *Analysis of Biliary Calculi.* Grind the calculus in a mortar with 10 ml. of ether. Filter.

- Filtrate I.
  Add an equal volume of 95 per cent alcohol[5] to the ether extract, allow the mixture to evaporate and examine for *cholesterol* crystals (Fig. 101). (For further tests see p. 300.)
- Residue I.
  (On paper and in mortar.)
  Treat with dilute hydrochloric acid and filter.
  - Filtrate II.
    Test for calcium, phosphates, and iron. Evaporate the remainder of the filtrate to dryness in a porcelain crucible and ignite. Dissolve the residue in dilute hydrochloric acid and make alkaline with ammonium hydroxide. A blue color indicates copper.
  - Residue II.
    (On paper and in mortar.)
    Wash with a little water.
    Dry the filter paper.
    Treat with 5 ml. of chloroform and filter.
    - Filtrate III.
      Bilirubin.
      (Red or yellow color)
    - Residue III.
      (On paper and in mortar.)
      Treat with 5 ml. of hot alcohol.
      Biliverdin.
      (Green color)

9. *Preparation of Taurine.* Add 50 ml. of concentrated hydrochloric acid to 150 ml. of bile in a casserole. Boil the mixture in the hood down to a volume

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[5] The alcohol is added because it is often found that crystallization from pure ether does not yield typical cholesterol crystals.

of about 50 ml., filter into a small evaporating dish to remove insoluble material, and concentrate the filtrate by boiling or on the water bath to a volume of about 10 ml. Filter the hot solution through a small filter into a 50-ml. graduated cylinder. If the volume is over 12 ml., return the filtrate to the evaporating dish, continue the evaporation, and filter a second time through the same filter. To the filtrate add 3 volumes of 95 per cent alcohol, mix, and cool in ice water for about 30 minutes. Filter off the crystals on a small funnel, allow to drain thoroughly, then transfer the crystals to a test tube and dissolve by warming in about 3 ml. of water. Add 5 volumes of alcohol and allow to stand until maximal crystallization has occurred. Filter off the pure crystals, wash with 5 ml. of alcohol, and allow to dry. Make the following tests upon the taurine just prepared:

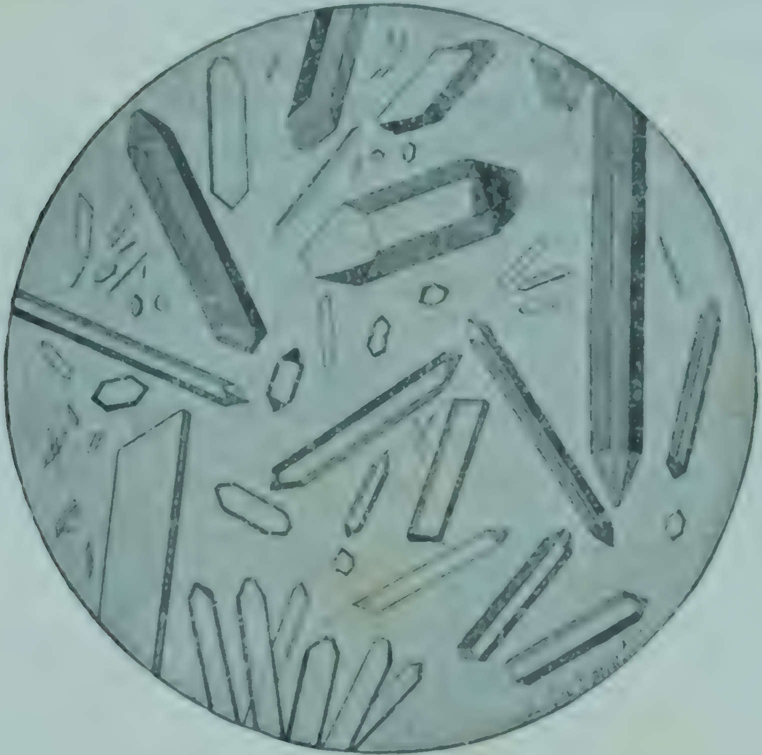

FIG. 102. TAURINE.

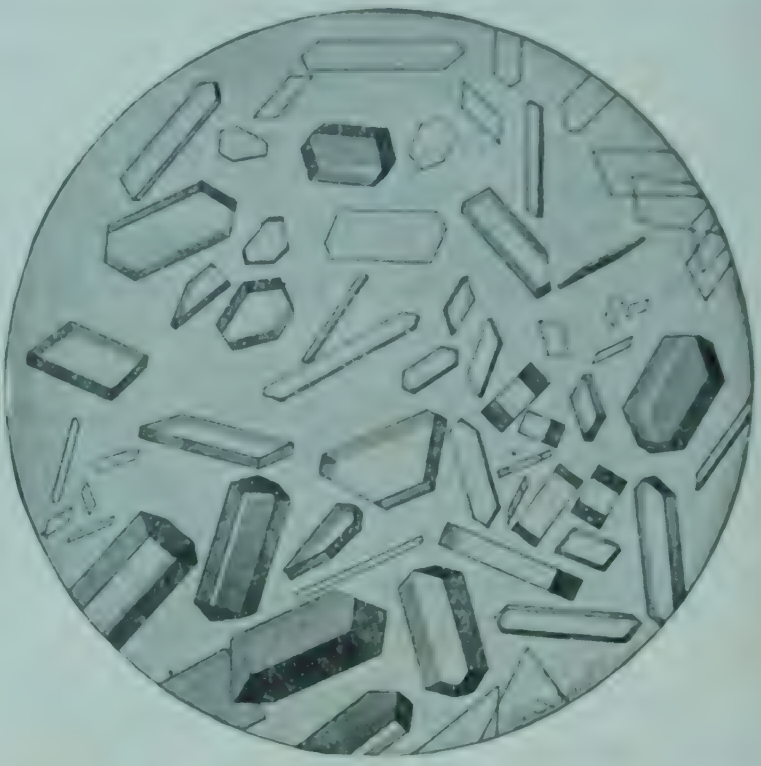

FIG. 103. GLYCINE. (SEE ALSO FIG. 38.)

a. Examine under the microscope. Compare with Fig. 102.

b. Solubility in water and alcohol.

c. Dissolve a little taurine in water and test this solution for sulfate by adding dilute hydrochloric acid and a few drops of barium chloride solution. Is any sulfate present? Boil the solution for a few minutes. Does taurine contain any ethereal sulfate (sulfate hydrolyzable by acid)? To another portion of taurine solution add sodium hydroxide, a few drops of lead acetate, and boil. Is any "lead-blackening" sulfur present?

d. Fuse a little taurine in a porcelain crucible with sodium carbonate-potassium nitrate "fusion mixture."[6] When the crucible contents are colorless, cool, dissolve carefully by the addition of dilute hydrochloric acid, filter into a test tube, and add barium chloride solution. What happens? Explain. What do these experiments indicate concerning the nature of the sulfur in taurine?

10. Preparation of Glycine. Concentrate the first alcoholic filtrate from Exp. 9 until no more alcohol remains. The glycine is present here in the form of a hydrochloride and may be liberated from this combination by the addition of freshly precipitated lead hydroxide or by lead hydroxide solution. Remove the lead from the filtrate by treatment with hydrogen sulfide. Filter

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[6] See Appendix.

and decolorize the filtrate by animal charcoal. Filter again, concentrate the filtrate, and set aside for crystallization. Glycine separates as colorless crystals (Fig. 103). See also pp. 131 and 132.

CHEMICAL EVALUATION OF THE FUNCTIONS OF THE LIVER

Many of the metabolic activities of the body are centered in the liver. Before discussing certain of these functions and the means for studying them in relation to liver disease, a résumé of the anatomy of the liver seems appropriate. It is necessary also to describe the major diseases of the liver in order to define more clearly the circumstances in which various chemical studies may be made and the response which may be expected.

The liver of a healthy adult may weight between 850 and 2600 g., with an average value of about 1600 g. Grossly, the liver consists of lobes varying in size and differing to some extent in the sources of afferent blood supplies. Each lobe contains a treelike framework of connective tissue which supports the parenchymal cells, blood vessels, and bile ducts. In its finer structure the liver consists of lobules, 1 to 2 mm. in diameter, composed of many secretory tubules formed by parenchymal cells surrounding a bile canaliculus. Blood reaches the lobules from the portal vein and the hepatic artery. The blood enters the sinusoids, spaces between cords or sheets of hepatic cells, and passes through them to drain into the central vein of the lobule.

The parenchymal cells, the most abundant and characteristic cells in the liver, are arranged so that each cell faces both a duct and a sinusoid containing blood, and have secretory and metabolic functions. Furthermore the secretory functions may be both exocrine and endocrine. In addition to the parenchymal cells, the liver contains large numbers of reticuloendothelial (Kupffer) cells which form a lining for the sinusoids. The Kupffer cells have phagocytic properties and are important in connection with hemoglobin breakdown and perhaps in immunity reactions.

DISEASES OF THE LIVER AND BILIARY TRACT

Disturbances of metabolism occurring in liver disease are largely the result of failure of the parenchymal cells to carry out vital functions, because of (1) infections or noxious agents; (2) decreased mass of functioning cells; (3) decreased blood supply; (4) impaired nutrition; and (5) reaction of other organs to liver damage, e.g., brain, kidney, pancreas, adrenal, gonads, and spleen.

Infectious disease of the liver, such as viral hepatitis, is characterized by degeneration and necrosis of parenchymal cells, and may be followed by complete disappearance of cells and destruction of the normal architecture of the lobule. However, regeneration of the parenchymal cells can occur rapidly, and may produce an astonishingly large mass of cells within as little as 24 to 48 hours. Formerly, viral hepatitis was often mistaken for a disease of the bile ducts and the term "catarrhal jaundice" in the older literature is a result of this confusion.

Deposition of fat in the liver may occur from overnutrition, from dietary deficiencies, or from the action of toxic substances. Though fatty liver resulting from overnutrition has little importance, deposition of fat caused by toxic substances is evidence of a serious disturbance of hepatocellular function. Among the nutritional deficiencies associated with fatty liver are those related to lack of betaine, choline, methionine, or other extrinsic sources of methyl groups. Low protein intake may also contribute to the production of fatty livers. Liver disease of purely nutritional origin is uncommon in the United States.

Liver damage may occur as a result of severe strains on metabolism associated with various other diseases, such as infectious mononucleosis, malaria, lobar pneumonia, typhoid fever, various anemias, syphilis, cholera, diabetes, and thyrotoxicosis.

Proliferation of the connective tissue of the liver may be of infectious, nutritional, toxic, hypoxic, or neoplastic etiology, or it may occur spontaneously because of diminished blood supply resulting from circulatory and other factors. The overgrowth of connective tissue in turn leads to disorganization of the liver structure and this again to further interference with the blood supply. The end result is a shrunken liver consisting largely of connective tissue and with a markedly decreased mass of parenchymal, reticuloendothelial, and vascular tissue. The designations portal cirrhosis, atrophic cirrhosis, and Laennec's cirrhosis have been used at various times to denote such scarred livers, although not all scarred livers conform to the pathologists' definition of Laennec's cirrhosis, which accounts for a large proportion of the liver disease seen in American hospitals. A less common form of cirrhosis, known as biliary cirrhosis, is found after prolonged biliary obstruction.

Obstruction of the bile ducts often causes jaundice which may be attributed erroneously to liver disease. It is essential to distinguish jaundice due to biliary obstruction from that caused by liver disease or excessive destruction of blood. Gallstones entering the common bile duct are the usual cause of biliary obstruction. Other causes include neoplastic disease of the ducts and carcinoma of the head of the pancreas. Stricture of the ducts may follow infection, surgical exploration, or other trauma. Disease of the gall bladder frequently is complicated by liver damage. The pancreas often shows evidence of being involved. Failure to establish the presence of biliary obstruction and to correct it may lead eventually to biliary cirrhosis.

Damage to the liver may be caused by a large number of chemicals and drugs. Carbon tetrachloride has been extensively used for production of experimental liver damage, and probably has been involved more often than realized as an insidious cause of clinical liver disease. Atophan, various sulfonamides, *p*-aminobenzoate, testosterone, arsenicals, and other drugs have been implicated. The effect on liver function, as measured by laboratory studies, resembles that observed in biliary obstruction rather than parenchymal liver disease.

Liver disease may affect the metabolism and functions of other organs, notably the brain and the kidneys. Impairment of kidney function commonly accompanies liver disease and may become a grave problem. The coexistence of hepatic and renal failure is often referred to as the *hepatorenal syndrome*.

THE CHEMICAL PHYSIOLOGY OF LIVER DISEASE[7]

Carbohydrate Metabolism and Liver Disease. The vital importance of the liver for maintenance of the blood glucose concentration is well established. Although hypoglycemia is not a common complication in patients suffering from acute parenchymal liver disease, it occurs in cirrhosis of the Laennec type with sufficient frequency to require that fasting blood glucose determinations be included in the study of such patients. Blood sugar concentrations as low as 25 mg. per 100 ml. are not uncommon. Glucose administered to patients with liver disease often causes a greater and more persistent rise in blood glucose than it does in healthy individuals; however, these findings are not of sufficient consistency to permit application as diagnostic or functional tests.

Decreased utilization of galactose in liver disease has provided the basis for one of the earlier tests of liver function.[8] The measurement of galactose excretion in urine originally used has been replaced by measurement of blood galactose concentrations.[9] An intravenous galactose-tolerance test has also been described,[10] by which the quantity of galactose removed per minute was found to be markedly decreased in liver disease.

Serum Cholesterol and Lipides in Liver Disease. The liver is the principal organ concerned with the metabolism and excretion of cholesterol. The serum lipides often show marked changes in diseases of the liver and biliary tract. Although serum cholesterol concentration and the partition between free and esterified cholesterol (see p. 580) is most frequently studied, neutral fat and especially phospholipide may also show marked changes. Relationships between free and esterified cholesterol and between free cholesterol and phospholipide which in health are maintained within narrow limits are subject to striking disturbances in severe parenchymal liver disease, where lipide concentrations often fall below the minimal levels observed in normal individuals of the same age. In viral hepatitis both the concentration of esterified cholesterol and the percentage of the total cholesterol esterified are lowered; if liver damage is severe, esterified cholesterol may become undetectable. Recovery is accompanied by rising concentrations of the esterified cholesterol in serum.

Cirrhosis of the Laennec type is also characterized by low serum lipide concentrations, especially when atrophy of the liver is extensive. Serum cholesterol concentrations of less than 100 mg. per 100 ml. are quite common. The proportion of esterified cholesterol also is lowered, although exceptions exist. Low phospholipide concentrations also are the rule.

Biliary obstruction regardless of cause is characterized by elevated concentrations of serum lipides. Extremely high concentrations, among the highest known to occur due to any cause, are encountered in patients with biliary obstruction of long duration or with biliary cirrhosis.[11] The reason is not known, since the bile does not appear to be an important route of lipide excretion.

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[7] For a detailed review, see Knisely, M. H., in *Trans. 10th Conference on Liver Injury*, edited by F. W. Hoffbauer, New York, Josiah Macy, Jr. Foundation, 1951.

[8] Bauer: *Wien. Med. Woch.*, **56**, 2538 (1906).

[9] Zieve, Hill, and Nesbitt: *J. Lab. Clin. Med.*, **36**, 705 (1950).

[10] Colcher, Patek, and Kendall: *J. Clin. Invest.*, **27**, 768 (1946).

[11] Ahrens *et al.*: *Medicine*, **29**, 299 (1950).

Some use is made of serum cholesterol analyses for differentiation of primarily parenchymal lesions from primarily biliary lesions in jaundiced patients. However, such analyses offer little information that cannot be obtained more easily and dependably by other methods. The finding of low concentrations and ratios of esterified to total cholesterol in a jaundiced patient is strong but not conclusive evidence for parenchymal liver involvement of severe degree. Esterified cholesterol measurements have their greatest usefulness where the more sensitive tests become maximally positive and there is still a substantial amount of functioning parenchymal tissue remaining. As an indication for determination of esterified cholesterol, the presence of jaundice or of a marked elevation of serum bilirubin serves reasonably well.

Information concerning bile acids in liver disease is scanty and unsatisfactory. Improvements in sensitivity of the methods for bile acid determination in serum and bile should overcome this deficiency. Methods available in the past for estimating bile acid concentrations in serum indicated that bile acid enters the blood stream to attain concentrations of 10 to 20 mg. per 100 ml. in the presence of biliary obstruction. Elevated values may occur also in liver disease affecting the parenchyma predominantly.[12]

Nitrogen Metabolism in Liver Disease. The metabolic transformation of amino acids in the liver by synthesis, transamination, etc. may be impaired by disease or injury to produce an abnormal pattern of amino acid content in the blood and urine.[13] Certain tests employed for the study of liver function have as their basis the impaired metabolism of amino acids; for example, the tyrosine-tolerance test of Bernhart and Schneider,[14] and, in its possible relation to glycine synthesis, the well-known hippuric acid test of Quick (see p. 915) may also be included here. Amino acids contribute to the characteristic elevation of blood nonprotein nitrogen content found in severe liver disease, along with urea, creatine, creatinine, uric acid, and ammonia, although the elevated blood NPN is mainly the result of impaired kidney function. Blood urea may not be elevated in proportion to creatinine and total NPN levels, possibly due to impairment of reactions involved in the synthesis of urea. Elevated creatine content is not entirely the result of renal failure. Interest in blood ammonia in liver disease has been revived by the finding of signs suggesting hepatic coma in patients suffering from cirrhosis who had received ammonia-containing ion-exchange resins.[15]

Plasma Proteins in Liver Disease. The liver has a dominant role in plasma protein synthesis, being the source of plasma albumin and fibrinogen (and probably other proteins associated with blood clotting) and contributing important components of the α- and β-globulin fractions. The liver is also involved in the synthesis of γ-globulins, although there is much evidence indicating that γ-globulin synthesis is largely extrahepatic.

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[12] Sherlock and Walshe: *Clin. Science*, **6**, 223 (1948).

[13] Dunn, Akawaie, Yeh, and Martin: *J. Clin. Invest.*, **29**, 302 (1950).

[14] Bernhart and Schneider: *Am. J. Med. Sci.*, **205**, 606 (1943).

[15] Gabudza, Phillips, and Davidson: *N. Eng. J. Med.*, **246**, 124 (1952).

The serum albumin level is lowered in cirrhosis, in viral hepatitis during its clinically active stages, in nutritional liver disease, and in neoplastic disease involving the liver. Many consider determination of serum albumin to be among the most dependable measurements available for establishing the presence of liver disease (numerous other causes of low albumin concentration usually can be excluded without difficulty) and for following its clinical course. For this purpose it is superior to total serum protein concentration because changes in albumin are commonly masked by an equal and simultaneous rise in globulin, so that total protein may remain unchanged or nearly so. The lowered concentration of serum albumin is one of the major factors responsible for the occurrence of flocculation in the cephalin-cholesterol flocculation and related tests.

An increase in γ-globulin accounts for much of the increase in total globulin of serum. Whereas in healthy individuals the γ-globulin content rarely exceeds 1.6 g. per 100 ml., in liver disease concentrations double this are common, and concentrations five or more times the maximal normal occur in some patients with hepatitis. The marked rise in γ-globulin occurring in liver disease is similar to that occurring in many other diseases, and may therefore be somewhat nonspecific. In many patients it is accompanied by an elevation in the level of β-globulin, associated with the appearance of electrophoretically abnormal components in both β and γ fractions.

Fibrinogen appears to remain within normal limits or to be decreased in liver disease; the α-globulin fraction also shows a tendency toward lower levels. This is particularly evident where some of the specific proteins included in this fraction are examined; for example, serum cholinesterase content is markedly decreased in many patients with liver disease, as may also be amylase, lipase, and esterase activities.

Of the procedures available for the study of liver disease which are based upon abnormality in plasma protein production or composition, the determination of plasma albumin and globulin levels are among the most useful. These methods are described in pp. 601 to 607. Some patients, especially those with liver damage of moderate degree, do not show significant change in serum albumin or total globulin concentrations. The quantitative measurement of γ-globulin concentration may offer some advantage in this connection and salting-out methods are available for this purpose. In addition, widely used methods for detecting changes in serum proteins of the type occurring in liver disease are the semiempirical flocculation and turbidity tests (see p. 595). For information concerning tests of this type and an analysis of their mechanisms, Saifer's review[16] may be consulted.

Measurement of Excretory Capacity of the Liver. Many substances, among them a number of dyes, are taken up by the liver and secreted into the bile with great rapidity. It has been found that in liver disease the rate of excretion may be lowered, and because of this the use of certain of these substances has been of great value in the measurement of liver function. Disodium phenoltetrabromophthalein sulfonate (sulfobromophthalein, sodium; bromsulfalein), introduced in 1925 by Rosenthal and White, has proved to be superior to numerous other substances tested for this purpose. The procedure for the bromsulfalein test for liver function is given on p. 598.

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[16] Saifer: *Am. J. Med.*, **13**, 730 (1952).

Detoxification Reactions of the Liver. Numerous studies have demonstrated that in liver disease various reactions associated with detoxification are impaired. Among these are included the synthesis of hippuric acid following administration of benzoate. This is discussed in detail on p. 915. An improved test employing *p*-aminobenzoate and based upon serum analysis rather than on urinary excretion has been described.[17] Conjugation of various substances with glucuronic acid has also been used as a basis for liver-function tests.[18]

Bile Pigment Metabolism in Liver Disease. Jaundice is such a conspicuous sign of liver damage or bile-duct blockage that it has attracted more than its share of attention, often to the neglect of other and more important aspects of liver or biliary-tract disease. The chemistry and metabolism of the bile pigments is discussed on p. 411. Methods for the determination of bile-pigment levels in serum (icteric index, Van den Bergh test, total serum bilirubin) are described on pp. 590 to 595, along with the interpretation of results in relation to liver disease. In general, rising serum bilirubin levels have unfavorable implications; falling values are characteristic of remission of liver disease or biliary obstruction. In the urine, bilirubin is normally present in such low concentrations as to be undetectable by ordinary methods; the presence of detectable amounts of bilirubin in urine is indicative of liver damage or biliary obstruction, particularly in the early stage of disease. After the presence of the disease has been established, urine bilirubin tests offer little useful information. The detection of bilirubin in urine is described on p. 836.

Bilirubin excreted into the intestinal canal is reduced in part to form urobilinogen, which may then be reabsorbed into the blood and again excreted through the bile and also into the urine. Normal urine contains detectable amounts of urobilinogen (see p. 813); impaired excretory ability of the liver leads to an increased urinary output unless liver damage is such that secretion is suppressed, or there is biliary obstruction. Increased urobilinogen in the urine is a characteristic finding in parenchymal liver disease. In the feces, urobilinogen (stercobilin) levels are increased in hemolytic anemia and lowered in biliary obstruction.

A summary of the relation between liver disease, bile-pigment metabolism, and other aspects of liver function is found in the accompanying table.

Other Chemical Manifestations of Liver Disease. Abnormalities in blood clotting in liver disease result from a combination of defects, including diminished prothrombin and accelerator globulin levels and

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[17] Deiss and Cohen: *J. Clin. Invest.*, **29**, 1014 (1950).

[18] Ottenberg, Wagreich, Bernstein, and Harrow: *Arch. Biochem.*, **2**, 63 (1943); Snapper and Saltzman: *Arch. Biochem.*, **24**, 1 (1950).

COMPARISON OF LIVER FUNCTION TESTS IN LIVER DISEASE

Syndrome	*Bilirubin*	*Tests for Parenchymal Involvement**	*Tests for Biliary Tract Involvement*
Viral hepatitis without jaundice	May be present in urine, and may increase slightly in serum. Urobilinogen may increase in urine.	BSF usually abnormal. CCF, TT, TF, one or more may be abnormal.	Normal
Viral hepatitis with jaundice	Increased in serum and urine. Urobilinogen generally increased in urine and feces but may be absent.	Abnormal	Mainly normal but may be abnormal.
"Toxic" hepatitis; cholangiolitic jaundice	Increased in serum and urine.	CCF, TT, TF, may be normal or abnormal; A/G usually normal.	Increased serum phosphatase, lipide. Similar to extrahepatic obstruction.
Laennec's cirrhosis	May or may not be abnormal in serum and urine. Urobilinogen variable.	BSF abnormal. CCF, TT, TF abnormal in about ¾ of the cases. A/G abnormal.	Variable. Phosphatase may be high.
Extrahepatic obstruction. *Partial*	Variable. Intermittent or continuous elevation.	Generally normal, but liver parenchyma may become injured and tests positive.	Variable with elevated serum phosphatase and lipide prevalent. Good response to vitamin K.
Complete	Extreme elevation. Very low fecal urobilinogen.	Same as preceding.	Elevated serum phosphatase and lipide. Good response to vitamin K.
Primary biliary cirrhosis	Bilirubin elevated. Urobilinogen variable.	BSF increased. A/G abnormal. TT increased. CCF increased in ½ of the cases.	Elevated phosphatase. Marked elevation of serum lipide, especially phospholipide.
Hemolytic jaundice	Total serum bilirubin moderately elevated; direct bilirubin normal or slightly increased. Urine bilirubin negative. Feces urobilinogen increased, urine urobilinogen often normal.	Seldom abnormal but may become so due to hypoxia or other complications.	Seldom abnormal. Pigment stones may cause biliary obstruction.

* Abbreviations: BSF, bromsulfalein; A, serum albumin; G, serum globulin; CCF, cephalin-cholesterol flocculation; TF, thymol flocculation; TT, thymol turbidity.

probably other factors.[19] The depletion of prothrombin in biliary obstruction is related to impaired vitamin K administration provided liver damage is not excessive. The evaluation of the clotting function of the blood is discussed in Chapter 22.

Liver disease may be associated with changes in blood and urine levels of such substances as citrate,[20] lactate, pyruvate, succinate, etc., but study of such changes has not yet reached the stage of general clinical application. Hormone metabolism may also be altered in liver disease; for example, both the conjugation of injected testosterone[21] and its excretion as 17-ketosteroid[22] are decreased in liver disease.

Disturbances of water and electrolyte balance are among the more significant and troublesome complications of liver disease. These are due in part to impairment of liver function and in part to the effect of liver disease on the kidneys and other organs. Among the blood analyses which are of clinical importance under these conditions are those for plasma proteins (p. 601), serum sodium (p. 649), and serum potassium (p. 652). Serum iron levels may also be altered in liver disease, being elevated in hepatitis[23] and in hemochromatosis; in the latter condition, the iron-binding capacity of the serum may be at the saturation level as compared with 14 to 69 per cent of capacity in cirrhosis and 28 to 59 per cent of capacity in normal individuals.[24]

Measurement of the alkaline phosphatase activity of serum (see p. 635) often assists in the differentiation of parenchymal liver disease from that due to obstruction and other lesions of the biliary tract. Biliary obstruction is characterized by a persistent increase in phosphatase activity to two or more times the maximum found in healthy individuals. This rise occurs regardless of the nature of the obstruction, whether due to calculus, stricture, or neoplasm. Elevation of serum alkaline phosphatase may provide one of the few clues to the presence of neoplastic growth in the liver.[25]

Elevated values are also found in toxic hepatitis of chemical origin, and in inflammatory disease of the bile ducts (cholangiolitic hepatitis), but are uncommon in cirrhosis of the Laennec type. The factors responsible for variation in serum phosphatase activity in liver disease are not clearly understood.

Serum cholinesterase activity (p. 638) is usually lowered in the presence of atrophy of or damage to the liver parenchyma, although not consistently. Serial measurement of cholinesterase activity appears to provide a useful method for following the course of liver disease, particularly that associated with deficient diets.

Selection of Procedures. The choice of methods for the study of disease of the liver or biliary tract will vary according to the type of information sought. The following outline shows the principal purposes for which chemical methods are applied together with a list of the procedures most likely to prove useful. A decision regarding the number of tests to be used requires experience and judgment. Usually those tests italicized will suffice for an initial study. To apply simultaneously the entire group of tests listed in any of the categories would seldom be justified.

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[19] For reviews, see Harrington *et al.*: *Bull. N. Eng. Med. Center*, **12**, 121 (1950); Stefanini: *Am. J. Med.*, **14**, 64 (1953).

[20] Sjostrom: *Acta chir. Scand.*, Supp. **49** (1947).

[21] Cantarow, Paschkis, Williams, and Havens: *Fed. Proc.*, **10**, 23 (1951).

[22] West: *J. Clin. Endocrin.*, **11**, 897 (1951).

[23] Peterson: *J. Lab. Clin. Med.*, **39**, 225 (1952).

[24] Gitlow, Beyers, and Colmore: *J. Lab. Clin. Med.*, **40**, 541 (1952).

[25] Mendelsohn and Bodansky: *Cancer*, **5**, 1 (1952).

1. Detection of liver damage in absence of jaundice, e.g., early or subclinical hepatitis: *urine bilirubin, bromsulfalein retention, direct and total serum bilirubin, flocculation tests (e.g., cephalin-cholesterol flocculation, thymol turbidity and flocculation), urine urobilinogen.*
2. Detection of residual liver damage, "recovery" stages of hepatitis, chronic hepatitis, chronic passive congestion, portal cirrhosis: *bromsulfalein, direct and total serum bilirubin, flocculation tests, serum albumin and globulin,* prothrombin, serum cholinesterase, urine urobilinogen, and coproporphyrin.
3. Following the course of the jaundiced patient suffering from parenchymatous disease; *direct and total serum bilirubin, flocculation tests, serum albumin and globulin, prothrombin.* In addition, if severe, *serum esterified cholesterol, cholinesterase.*
4. Differentiation of jaundice due to biliary disease from that due to parenchymatous disease: *serum alkaline phosphatase, cephalin-cholesterol flocculation and thymol turbidity,* galactose tolerance, prothrombin, feces urobilinogen.
5. Differentiation of extrahepatic biliary obstruction due to calculus from that due to neoplasm, stricture, etc.: *feces urobilinogen.*
6. For following the course of the surgical patient with disease of the biliary tract: *plasma prothrombin, phosphatase, direct and total serum bilirubin, albumin and globulin, electrolytes, blood urea N or NPN,* serum lipides.
7. Differentiation of hemolytic jaundice: *direct and total serum bilirubin, feces urobilinogen,* erythrocyte fragility, reticulocyte count.

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19

Intestinal Absorption

Mechanism of Absorption. Absorption is primarily a function of the small intestine. Very little absorption takes place from the stomach, and the process is nearly complete before the colon is reached. The small intestine is particularly adapted to carrying on this process by virtue of its great length (about 9 meters in man) and by the structure of its mucous membrane. The surface of the latter is greatly increased (to a total area of about 10 square meters) by the presence of folds and of fingerlike processes called villi. The latter possess a rich blood supply, lymph spaces called lacteals, and muscle fibers whose contractions mechanically assist absorption. The mucous membrane through which substances must pass to enter the blood or lymph is extremely thin, but it must not be forgotten that it consists of living cells.

Substances in solution tend to distribute themselves uniformly throughout the solvent, and this tendency, which we measure as osmotic pressure, is a powerful force. Undoubtedly osmosis may play some role in the absorption of certain substances which may be present in the intestine in far higher concentrations than in the blood. There is much evidence, however, that the process is rarely one of simple diffusion.

The facts with regard to the absorption of ionic substances (salts, amino acids, etc.) have been made much more intelligible by the development of the Donnan theory of membrane equilibria. This explains how it is possible for ionic diffusion to be more or less selective and how certain ions may pass into the blood although present there in higher concentration than in the intestinal lumen.

Even with this extension, however, it is not yet possible to explain many phenomena of absorption on a purely physical basis. Not only do the regulatory powers of the cell and its changing structure influence the course of diffusion processes but it seems probable that certain substances in their passage enter into chemical combination with constituents of the protoplasm thus making possible different types of selective transportation. Furthermore, white blood cells actually migrate back and forth between the lymph and the lumen of the intestine. There is evidence that certain of these may actually engulf particles of iron compounds and fat droplets. One of their functions may thus be to assist by phagocytosis in the absorption of difficultly soluble substances. Their number is hardly adequate to permit their playing a large role in the absorption of foodstuffs; however, their accumulation in the intestinal mucosa during digestion would indicate that their function is an important one.

The intestinal mucous membrane further possesses the power of excreting certain substances into the gut. Sucrose injected into the blood stream appears in part in the duodenum, and calcium salts are eliminated by the mucosa of the large intestine. The glands of the mucosa secrete a digestive fluid, the intestinal juice, and the cells themselves contain enzymes such as proteinases and peptidases, carbohydrases, and phosphatases which help to prevent the passage of incompletely decomposed proteins, carbohydrates, and organic phosphates into the blood.

Because of the many factors involved, the study of intestinal absorption offers certain difficulties. Investigation of the processes concerned helps, however, to throw light on permeability and other fundamental properties of protoplasm. It is further of the greatest practical importance to understand the factors governing the absorption of the many essential foodstuffs, and to determine the conditions under which there is a loss of the great protective power of the intestinal mucosa to detoxicate or prevent the absorption of toxic substances, which may be ingested or produced during digestion or putrefaction.

Mineral oil has been shown to interfere with the absorption of fat-soluble vitamins and various digestion products. Certain adsorbing agents used therapeutically also interfere, and all cathartics interfere because of the reduced time the products of digestion remain in the intestine.

Absorption of Carbohydrates. Carbohydrates are absorbed almost entirely as the simple monosaccharides, glucose, fructose, galactose, mannose, and the pentoses, each having a characteristic absorption rate. Cori showed that galactose was absorbed most rapidly. Next came glucose and fructose. L-Arabinose had the slowest absorption rate, less than one-tenth that of galactose and glucose. There is considerable evidence favoring the view that a hexokinase-catalyzed phosphorylation takes place in the intestinal absorption of glucose, followed by a dephosphorylation of the sugar by phosphatases in the intestine before it enters the circulation. Of the disaccharides, lactose is least readily hydrolyzed in the intestine and some of it occasionally reaches the blood as such. In these cases it is eliminated unchanged in the urine. Sucrose entering the blood is also excreted as such. When strong sugar solutions enter the bowel they are diluted by the intestinal secretion until the concentration is reduced to a point favorable for absorption; i.e., when the osmotic concentration equals that of the blood plasma. Lactose, because of its slow digestion and absorption, reaches a much lower portion of the bowel than other sugars and is thus more effective in promoting the growth of acidophilic rather than putrefactive bacteria.

The simple sugars entering the blood are quite rapidly removed therefrom by the liver and other tissues for oxidative and storage purposes. After the ingestion of 100 g. of glucose a maximum blood-sugar value of about 0.15 per cent is reached in less than an hour, the normal figure of about 0.1 per cent being restored by the end of two hours. This rapid absorption of glucose and its subsequent oxidation in the tissues to yield energy are of importance to persons engaged in prolonged athletic competition or in other fatiguing activities.

If the intestinal mucous membrane is destroyed, or poisoned by such a substance as sodium iodoacetate, it acts like an ordinary permeable membrane and sugars pass through it according to ordinary laws of diffusion. Thus the pentoses pass through more rapidly than the hexoses. With the normal living membrane, however, the hexoses show more rapid absorption. Apparently the hexoses are combined with phosphate in passing through the cells of the mucous membrane. The formation of this compound greatly speeds up the absorption process and makes possible absorption even from a sugar solution of lower concentration than the blood. The intervention of chemical mechanisms of the living cell also makes possible a certain selectivity in absorption, and an adaptation of the mucous membrane in the direction of self-protection of the body against rapid absorption of toxic substances.

Absorption of Protein. Protein is absorbed for the most part in the form of individual amino acids. The amino acid content of both portal and systemic blood rises after a meal. The amino acids of the diet entering the body are carried by the blood to all the tissues of the body, where they rapidly become incorporated into the metabolic processes of the cells and become indistinguishable from the amino acids already present unless they have been labeled in some way, as with isotopic nitrogen. Thus the dietary amino acids are not to be regarded as somewhat of a surplus, as they were at one time, to be drawn upon or oxidized as the need arose, but rather as a daily contribution to the general processes of nitrogen metabolism in the body (see Chapter 33).

Whereas proteins are absorbed mainly as amino acids, it is nevertheless true that certain molecules larger than the amino acid molecules, e.g., peptides or polypeptides, may at times be absorbed. *Strepogenin*, a peptidelike substance, is definitely absorbed.[1] With the aid of immunological methods it has also been established that certain unsplit proteins may be absorbed unchanged.

A variable and sometimes significant proportion of the dietary amino acids escape absorption and are metabolized by the bacteria of the intestinal tract. The products of this action may be absorbed and appear in the blood or urine. The relatively high ammonia content of portal blood is attributed to these processes, as is the indican content of the urine. The significance of such intestinal putrefaction is discussed in Chapter 20.

Incompletely decomposed protein may sometimes be absorbed, as is indicated by the production in certain individuals of anaphylactic reactions (cutaneous eruptions, asthma, etc.) following the ingestion of particular types of protein. Such reactions are not produced by completely hydrolyzed proteins.

Absorption of Fat. The exact mechanism by which fat is absorbed is the subject of considerable controversy. Two theories have been proposed, namely, the *lipolytic* and the *partition theory*, and each of these possesses certain merits. According to the lipolytic theory as defined by Bloor as well as by Verzár, a complete hydrolysis of the ingested fat occurs in the gastrointestinal tract as a preliminary to absorption. Glycerol formed as a result of such hydrolysis is readily soluble in water;

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[1] Woolley: *Federation Proc.*, 6, 424 (1947).

there is considerable experimental evidence that this compound can be readily and completely absorbed. On the other hand, the fatty acids resulting from hydrolysis of the neutral fats are insoluble in water, although their soaps dissolve quite readily. However, the pH of the small intestine is such as to preclude the presence of appreciable amounts of soap. Since it is known that most fats are almost completely digested, some mechanism is required to transport the water-insoluble fatty acids through the gut wall if soaps are excluded. The mechanism seems to involve the presence of bile concomitant with the fat. Under such conditions an emulsion is formed which is reflected in the milky appearance (chyle) of the lymph in the mesenteric vessels during the absorption of fat. This condition does not obtain in the absence of bile. Bile is believed to facilitate digestion both by activating pancreatic lipase and also by rendering the fatty acids soluble in the aqueous medium. The latter change is mediated by the bile salts, which lower the surface tension. It has been postulated that the bile salts combine with the fatty acids to form compounds known as *choleic acids*. Wieland and others have demonstrated that such compounds, produced in the test tube, have definite melting points, and that there are fixed ratios of fatty acids to bile acids (usually 1:8 in the case of C_{16}-C_{18} acids). The importance of this mechanism remains questionable, however, since the only naturally occurring bile acid which is active is deoxycholic acid, and this is a minor component in most biles. Moreover, only the unconjugated form is active; but the conjugated acids (glycodeoxycholic and taurodeoxycholic acids) represent the forms occurring in the bile. The fact that such coordination compounds as choleic acids exist is however an intriguing one, and these compounds may be related to fat absorption.

According to the partition theory, as proposed by Frazer, fats are only partially hydrolyzed in the gut, with the resultant formation of some fatty acids, and also mono- and diglycerides. The bulk of the fat is in the form of triglycerides. These lower glycerides and fatty acids, together with bile salts, form a stable emulsion which consists largely of triglyceride fats. This particulate matter, when the droplets are of a size of 0.5μ or less, is directly absorbable through the fine canals in the outer border of the intestinal cells. The results of Mattson *et al.* (see Chapter 33) tend to support this hypothesis. They showed that the intestinal contents of rats, removed three hours after the animals had been fed a mixture of partially hydrogenated cottonseed and soybean oils, contained principally unhydrolyzed triglycerides, diglycerides, and monoglycerides, with only 15 per cent of free fatty acids. Frazer is of the opinion that triglycerides of short-chain fatty acids, whose acids are water-soluble, may be completely hydrolyzed in the intestine.

There are ample experimental data which demonstrate that, irrespective of the fatty material fed, neutral fat appears in the intestinal lymph. According to the Verzár school, the products of fat digestion are recombined in the intestinal mucosa, possibly through the intermediation of phospholipide. Frazer also suggests that considerable modification and resynthesis of triglycerides may occur in the intestinal cells. The bile acids set free are returned to the lumen of the gut to assist in the absorption of additional fat. Although new phospholipides may be formed in the intestinal cells, as demonstrated by the incorporation of the unnatural fatty acid *elaidic acid* in the intestinal phospholipides after the feeding of *trielaidin* (Sinclair), considerable quantitative data are now available which indicate that the phospholipides are not obligatory intermediates in fat synthesis.

By means of tests (rats) in which decanoic and palmitic acids labeled with C^{14} in the carboxyl position were fed, it was learned that short-chain fatty acids are transported mainly by the portal pathway, and long-chain saturated fatty acids via lymph.[2]

After resynthesis, the greater part of the fat first enters the lacteals of the intestinal villi and then the lymphatics, forming an emulsion (the chyle) which is carried via the thoracic duct to the jugular vein. An increase in the fat content of the blood and lymph following a meal ("fat tide") is readily demonstrated, the fat appearing in the form of minute globules called chylomicrons. The fat of the diet is therefore unique among the other components of the diet in that in large part it bypasses the liver. The significance of this anatomical arrangement is not clear, but it may possibly be related to the recognized ability of liver tissue to oxidize fatty acids rapidly to the stage of acetoacetic acid and β-hydroxybutyric acid.

Absorption of Sterols. Cholesterol, the most important of the sterols, like the fatty acids forms compounds with the bile acids which facilitate its absorption (see Chapter 18). Unabsorbed cholesterol is reduced to coprosterol. It is uncertain whether this change to coprosterol is brought about solely through bacterial action. The plant sterol phytosterol is not absorbed and hence cannot become a source of cholesterol in the animal body. Ergosterol is said to be absorbed but slightly if at all; but the irradiated form (calciferol) is more readily absorbed. Apparently the absorption of sterols is very specific, so that even isomerism may alter absorbability, and saturation of unsaturated bonds may change a sterol from a readily absorbable substance to one completely unabsorbable. Of the many sterols found in plant or animal foods, the only one which is absorbed in the human intestine, aside from the D vitamins, is cholesterol. Certain forms of vitamin D are absorbed to different degrees in different species (see Chapter 35).

Absorption of Inorganic Salts. The selective nature of absorption applies even to inorganic salts. Thus sulfates are much less readily absorbed than chlorides and tend to withdraw water from the blood. For this and other reasons the sulfates have a cathartic effect. The absorption of calcium and phosphorus is of especial interest because of its relation to the development of rickets and because this absorption can be so profoundly affected by minute amounts of antirachitic vitamin and hence by ultraviolet radiation. The acidity of the intestinal contents is also of importance for the absorption of the relatively insoluble salts of calcium. The most rapid rate of absorption of calcium after the administration of $CaCl_2$ solution containing Ca^{45} has been found to occur within the first 2 to 4 hours. This absorption was principally from the proximal part of the small intestine.[3] Dietary iron appears to be absorbed in nutritionally significant amounts only when it is in the ionized inorganic form, and clinical and experimental evidence indicates that ferrous iron is much more available for absorption than ferric iron. Calcium, magnesium, phosphorus, and iron are excreted to a considerable extent by the intestinal mucosa. Hence a study of their absorption at different levels of the intestinal tract is required for an understanding of the factors involved. The absorptive power of the colon for food substances is relatively low.

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[2] Kiyasu, Bloom, and Chaikoff: *J. Biol. Chem.*, **199**, 415 (1952).

[3] Harrison and Harrison: *J. Biol. Chem.*, **188**, 83 (1951).

Methods of Studying Absorption. Much of our information on absorption has been obtained from the study of isolated intestinal loops which retain their nerve and blood supply. Solutions can be injected into such loops and the contents removed at any time for analysis. The relative rates of absorption of various substances can thus be determined under controlled conditions. Small animals such as the rat may be sacrificed after the ingestion of test substances, the intestinal tract removed, washed out, and the washings then analyzed to establish the extent of absorption.

Histological examination of the mucosa of animals killed after a meal has shown the presence of fat globules in the cells.

By establishing fistulas of the intestine, the course of absorption in different parts of the tract has been studied.

Analysis of the blood and lymph gives information of great importance relative to the nature of the products entering the blood stream. Urine analyses show the rapidity with which soluble inorganic salts are absorbed. Perfusion experiments on the intestine are little used because a normal mucous membrane is difficult to maintain. Studies on absorption from the stomach may be made with a stomach tube, and a similar procedure may give some information as to absorption from the colon. Fecal analyses show the completeness of digestion and absorption of various substances present in the food.

Animals may also be fed a diet containing a definite amount of a nonabsorbable substance such as iron oxide, or a labeled component such as elaidic acid or compounds containing the isotopes of hydrogen, phosphorus, nitrogen, iron, or carbon. By suitable analyses of the intestinal contents and the other parts of the animal body the rate and extent of absorption may be evaluated. The use of isotopes in particular appears to offer great promise in elucidating the mechanism of absorption. For a discussion of isotopes, see Chapter 32, p. 970.

EXPERIMENTS ON ABSORPTION

1. ***Experiment to Show the Action of Bile Salt Solutions on Fatty Acids and Cholesterol.*** **Prepare five test tubes as follows:**

a. **Five ml. of buffer solution pH 7 + 2 ml. of a 10 per cent solution of bile salts + 1 ml. of a 1 per cent solution of oleic acid in alcohol.**

b. **Five ml. of buffer solution pH 7 + 2 ml. of water + 1 ml. of 1 per cent oleic acid solution.**

c. **Five ml. of buffer solution pH 9 + 2 ml. of water + 1 ml. of 1 per cent oleic acid solution.**

d. **Five ml. of water + 2 ml. of 10 per cent bile salt solution + 1 ml. of a 0.05 per cent solution of cholesterol.**

e. **Five ml. of water + 2 ml. of water + 1 ml. of cholesterol solution.**

Place all tubes in a water bath at 40° C. for a few minutes and observe the tubes for turbidity.

Fatty acids form a clear soap solution only at pH 9 or higher. With bile salts a clear and diffusible solution is formed even below pH 7. Bile salts also have a similar action on cholesterol. This is important also in connection with the excretion of cholesterol in the bile.

2. Determination of Rapidity of Absorption of Sugars from the Entire Gastrointestinal Tract (Method of Cori): Principle. Animals are given sugar solutions by a stomach tube. After a suitable time has elapsed the animals are killed, the entire gastrointestinal tract removed, and the total sugar remaining unabsorbed determined.

Procedure. Rats two to three months old and weighing from 120 to 180 g. are weighed. They are then placed in small wire-screen cages with screen bottoms so that there is no access to feces. For 48 hours they are given water but no food. The rats are again weighed and are then fed the solutions to be tested, usually 1.25 to 2.5 ml. of 25 to 80 per cent sugar solutions. These are introduced by means of stomach tubes consisting of Nos. 4 to 5 urethral catheters softened by plunging for a moment into boiling water. A small mouthpiece is used and the catheter marked to indicate the depth to which it should be introduced. A syringe of the Record type with a needle to connect with the catheter is used to inject the fluid. If diarrhea is caused the experiment is discarded.

An animal is killed at each hourly interval. The entire gastrointestinal tract is removed, slit open, and washed thoroughly with water making up to a volume of nearly 500 ml. A small amount of dialyzed iron is added and then a little sodium sulfate to precipitate it, along with interfering substances. The sugar is determined by the Benedict method or some other method. Blood sugar may also be determined by the Hagedorn-Jensen method or other micro method. Sugar may also be determined in the urine. (In another experiment it may be shown by analysis of the gastric contents that a dilution of the sugar solution takes place in the stomach.) The amount of sugar absorbed per 100 g. of body weight of the animals is calculated. An absorption curve may also be plotted from the results obtained with similar animals at 1, 2, 3, 4, etc., hours. Cori found that the rate of absorption of hexoses is independent of the concentration, and the rates of absorption of some monosaccharides are in the following order: galactose > glucose > fructose > mannose > xylose > arabinose. No glucose appeared in the urine but about 50 per cent of the galactose was excreted by this channel.

If a soluble ferric salt such as ferric ammonium citrate is added to the sugar solutions it is possible to determine in what parts of the tract digestion and absorption of carbohydrates, etc., more particularly occur (see the experiment below).

The tolerance of animals for sugars injected intravenously may also be determined and an idea obtained as to the rapidity with which sugars are absorbed when injected intravenously as compared with absorption from the intestine. Cori found the tolerance for glucose given intravenously to be from 2.2 to 2.5 g. per kg. of body weight per hour.

3. Influence of Carbohydrates on the Utilization of Calcium and Phosphorus (Method of Bergeim[4]): Principle. To a standard diet is added a definite

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[4] Bergeim: *J. Biol. Chem.*, **70**, 29 (1926); Gallup: *J. Biol. Chem.*, **76**, 43 (1928); Heller, Breedlove, and Likely: *J. Biol. Chem.*, **79**, 275 (1928). For use of silica see Greenwald and Gross: *J. Biol. Chem.*, **66**, 185 (1925); **82**, 505 (1929); Gallup: *J. Biol. Chem.*, **81**, 321 (1929).

For use of the iron method in the study of digestion and absorption in different parts of proportion of iron oxide and of the carbohydrate whose effect it is desired to study. The ratios of calcium and phosphorus to iron are determined for foods and feces and the percentage absorption calculated. The accurate separation of feces of the experimental period is not necessary.

***Procedure.* Feed two or more albino rats (about 60 g. in weight) on a phosphorus-low and calcium-high diet (whole yellow corn 76, wheat gluten 20, calcium carbonate 3, sodium chloride 1, and c.p. ferric oxide 0.2) for about three weeks. Put in separate cages with screen bottoms. Collect feces for a five-day period. Then modify the diet of the animals by substituting glucose for 30 parts of corn in one case, and an equal amount of lactose in the other. After a two-day interval, collect the feces for a period of four days. Then exchange diets so that the first animal gets lactose and the second glucose. After a two-day interval collect feces again over a four-day period. All feces need not be collected. Those contaminated with urine are discarded.**

About 0.5 g. (not weighed) of feces from each collection is ashed, preferably in a 35-ml. silica crucible at a moderate temperature. Add a few drops of nitric acid to the residue and heat again to destroy the last carbon. Add 10 ml. of 15 per cent hydrochloric acid and heat until the ash is dissolved. Wash into a flask with water to make about 35 ml. Ash 2 g. of food and dissolve the ash in the same way. Determine iron, calcium, and phosphorus in the ash solutions by standard methods (see Chapters 23 and 31) and record the number of mg. of each in 1 ml. of ash solution.

CALCULATION. Calculate the ratios Ca/Fe and P/Fe for food and feces, and calculate percentage absorption or utilization of Ca and P. For example, if the ratio Ca/Fe for food is 10:1 and in feces 4:1, unabsorbed Ca is 4/10 or 40 per cent, and utilization is 100 − 40 = 60 per cent.

Lactose promotes calcium absorption by creating an acid medium (lactic acid) in the intestines. Glucose has little effect. Vitamin D markedly improves absorption of Ca and P.

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the gastrointestinal tract see Bergeim: *J. Biol. Chem.*, **70,** 47 (1926); *Arch. Internal Med.*, **37,** 110 (1926).

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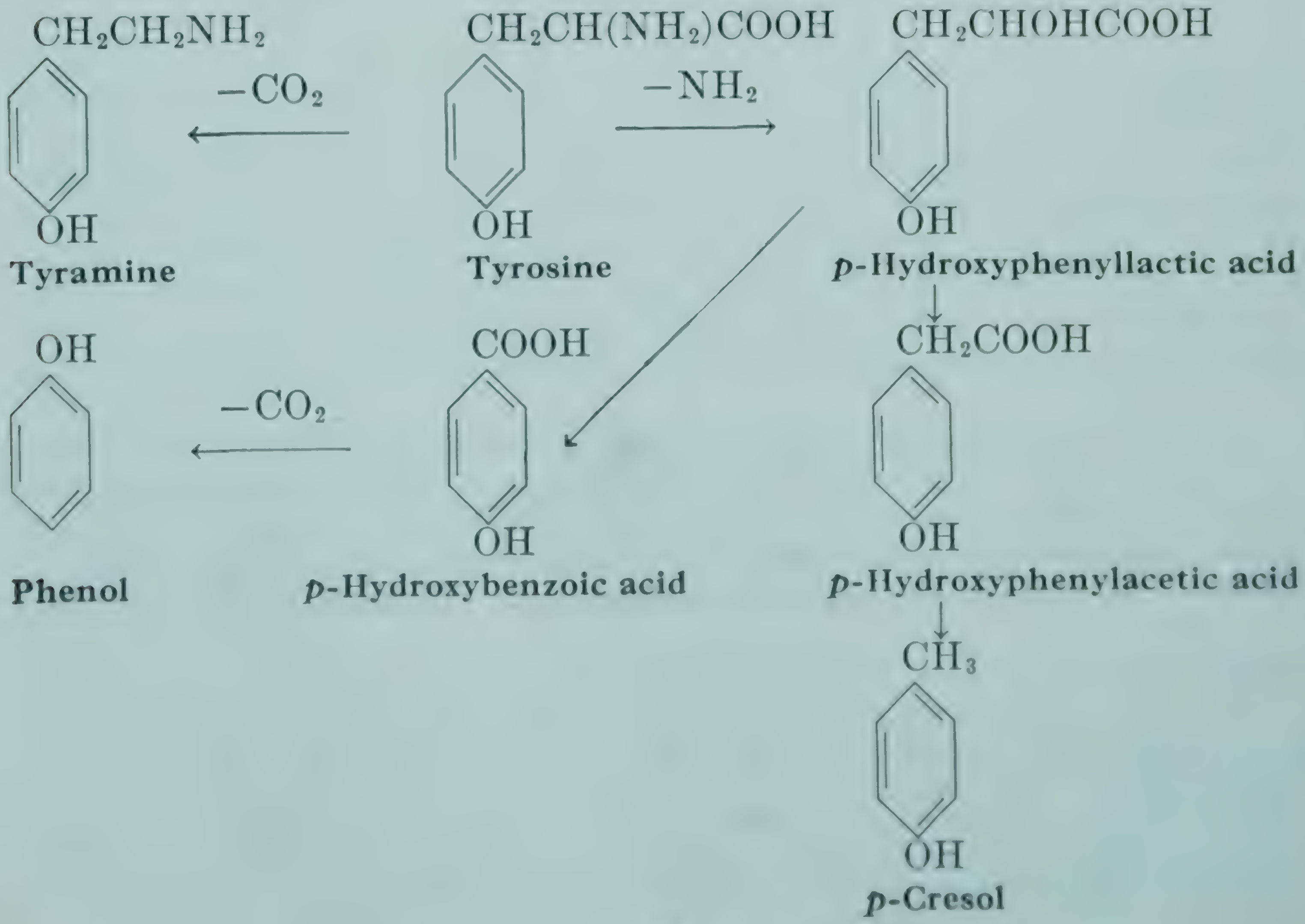
Putrefaction, Detoxication, and Conjugation

The food residue and the digestive and other secretions as they pass into the lower ileum and colon are acted upon by the bacterial flora which become permanently established in man during the first few days of life. The extent of bacterial activity is indicated by the fact that nearly one-third of the solid matter of normal feces is made up of bacteria.

The metabolic products of the intestinal bacteria are for the most part harmless, and some are actually useful; but a few, especially those arising from proteins and amino acids, are toxic in varying degrees. In the past it was believed that an excess production of these products, such as might occur in constipation, caused a condition of autointoxication characterized by malaise, headache, and irritability. Probably mechanical factors as well as emotional and psychic influences often account for some of these disagreeable effects attributed to constipation.

When amino acids are acted upon by bacterial enzymes, they undergo decarboxylation, deamination, and other reactions, with the result that abnormal products are formed. Some of these are toxic and are handled in the body by chemical mechanisms that are generally regarded as detoxifying. The variety of products that may result from an amino acid is illustrated by tyrosine.


CH2CH2NH2
OH
Tyramine
−CO2
CH2CH(NH2)COOH
OH
Tyrosine
−NH2
CH2CHOHCOOH
OH
p-Hydroxyphenyllactic acid
OH
Phenol
−CO2
COOH
OH
p-Hydroxybenzoic acid
CH2COOH
OH
p-Hydroxyphenylacetic acid
CH3
OH
p-Cresol

Action of Products of Putrefaction. Of the various types of compounds formed by bacteria acting on amino acids, the amines are physiologically the most active. Tyramine, which is formed from tyrosine, is a strong vasoconstrictor and like epinephrine, to which it is closely related structurally, it elevates blood pressure. Histamine, on the contrary, causes capillary dilatation. Although this compound may be formed by bacterial action, it is also normally produced by the body to serve as a stimulus for the secretion of hydrochloric acid in the stomach. In anaphylactic shock large amounts of histamine are released into the circulation. Since tryptamine is a product of putrefaction, it is interesting to recall that when platelets disintegrate during the clotting reaction, 5-hydroxytryptamine is produced.[1] This compound is a strong vasoconstrictor and probably plays an important role in hemostasis. Of the aliphatic amines, two are well known: pentamethylenediamine (cadaverine) and tetramethylenediamine (putrescine), which are derived from lysine and arginine respectively. Since they were originally isolated from putrefying flesh, they were classed as ptomaines. In spite of their offensive names, they are relatively harmless. The condition known as ptomaine poisoning is due to bacterial toxins and not to these amines.

Of the putrefactive products other than amines, indole and skatole, which are derived from tryptophan, have received much attention. Both possess a disagreeable odor and are mainly responsible for the characteristic odor of feces. Only a small fraction of the indole and skatole formed in the intestines is absorbed, as indicated by the finding that the daily excretion of indican (indoxyl potassium sulfate) rarely exceeds 10 to 20 mg. It is improbable that such small amounts have any deleterious effects. Phenol, *p*-cresol, and allied compounds formed from aromatic amino acids are toxic, but the quantities formed and absorbed are rarely large enough to be harmful provided the liver and kidneys function normally. Total phenol excretion by normal men averages about 0.2 g. per day.

Some of the bacterial products formed in the intestines are distinctly useful to the body. It is likely that part of the vitamin K required by the organism comes from the action of *E. coli* and other bacteria. It is known that various other vitamins such as biotin and thiamine are synthesized by bacteria, and that these can be utilized by the body. The bacterial flora may therefore be regarded as an auxiliary factor in nutrition. The benefits thus accruing are likely to outweigh the harm that may come from the toxic putrefactive products.

DETOXICATION

Though it is well known that the body can and does develop means of defense against microorganisms and their toxins, which usually are proteins, there is no convincing proof that it has evolved mechanisms specifically intended to detoxify either the products resulting from putrefaction in the intestines or the myriads of compounds and drugs that are ingested and absorbed.

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[1] Rapport, Green, and Page: *J. Biol. Chem.*, **180**, 961 (1949).

In general, all products of exogenous origin that enter the body may be divided into three major classes: (1) Substances which are normally utilized for energy, structure, or function. All may be broadly considered as food. (2) Substances which, in the doses ingested, alter physiological function. If the change in function is beneficial, the substance producing it is considered to be a drug; whereas if the alteration is harmful, the substance is considered to be a poison. (3) Substances which pass through the body unaltered and cause little or no change in function. Water is the most important example, although in a strict sense it may be regarded as a food.

In categories as broad as these, many instances of overlapping as well as difficulties of interpretation arise. Ethyl alcohol may be regarded as a food, a drug, or a poison. Similarly, nicotinic acid, being a vitamin, is a food; but it may also act as a drug and in large doses as a poison.

Although the protection against noxious substances is probably largely accidental rather than by design, detoxication nevertheless plays an important physiological role. It begins in the intestines. Thus, the enzyme histaminase, found in the alimentary tract, is probably responsible for the destruction of relatively large amounts of histamine. The intestinal wall is coordinated in the line of defense against poisons, since it acts as a barrier to various harmful substances. Many toxic substances that pass the intestinal walls are removed by the liver. It is not surprising therefore that this organ, which has as one of its functions that of a chemical watchdog, possesses many of the mechanisms which are usually designated as detoxication reactions. The principal reactions, namely, oxidation, reduction, hydrolysis, and conjugation, which the body normally employs for the destruction, detoxication, and elimination of a foreign compound are not essentially different from those employed on normal metabolites. Many foreign substances are only partially metabolized, thereby leaving a chemical clinker or residuum which often serves as a valuable source of information concerning metabolic pathways.

Oxidation, Reduction, and Hydrolysis. The first attempt of the body to protect itself against a toxic compound is to destroy it by oxidation. Many substances such as ethyl alcohol are completely burned to carbon dioxide and water. Sometimes the intermediary oxidation products are more toxic than the original compound, as illustrated by methyl alcohol, which yields formaldehyde and formic acid.

The oxidation of aromatic compounds is of great physiological importance. Benzene itself as well as many simple aromatic compounds such as benzoic acid and phenol are fairly resistant to oxidation. Certain side chains, particularly the groups $CH_2CH(NH_2)COOH$, which occurs in phenylalanine, and $CH_2CO{\cdot}COOH$ render the aromatic nucleus completely oxidizable. This suggests that oxidative deamination is the initial step in the metabolism of amino acids.

$$\underset{\textbf{Phenylalanine}}{C_6H_5CH_2CH(NH_2)COOH} + O \rightarrow \underset{\textbf{Phenylpyruvic acid}}{C_6H_5CH_2COCOOH} + NH_3$$

Of particular interest is the oxidation of phenyl-substituted fatty acids. Oxidation takes place on the β-carbon atom, and the side chain is progressively reduced by two carbon atoms, so that ultimately the aromatic acids having a side chain with an odd number of carbon atoms yield benzoic acid, and those with an even number yield phenylacetic acid.

$$\underset{\textbf{Phenylvaleric acid}}{C_6H_5CH_2CH_2CH_2CH_2COOH} \rightarrow \underset{\textbf{Phenylpropionic acid}}{C_6H_5CH_2CH_2COOH} \rightarrow \underset{\textbf{Benzoic acid}}{C_6H_5COOH}$$

$$\underset{\textbf{Phenylbutyric acid}}{C_6H_5CH_2CH_2CH_2COOH} \rightarrow \underset{\textbf{Phenylacetic acid}}{C_6H_5CH_2COOH} + \underset{\textbf{Acetic acid}}{CH_3COOH}$$

From these observations Knoop formulated the hypothesis that normal fatty acids are likewise catabolized by β-oxidation (see Chapter 33).

The finding[2] that the depancreatized dog oxidizes phenylbutyric acid to phenylacetic as readily as a normal dog is interesting because it was the first evidence suggesting that the oxidation of butyric acid was not impaired in diabetes.

Reduction and hydrolysis are encountered infrequently in the metabolism of foreign compounds. One example of reduction is the conversion of chloral hydrate to trichloroethyl alcohol, which is then combined with glucuronic acid. Glucosides such as the digitalis compounds probably undergo hydrolysis with the liberation of a sugar and an aglycone as a step in their metabolism and elimination from the body.

The conversion of cyanide to thiocyanate may be regarded as an important detoxication mechanism. This is indicated by the wide distribution in various tissues of the enzyme rhodanese, which is responsible for changing cyanides to thiocyanate, and also by the relatively high concentration of thiocyanates in the blood (over 1 mg. per 100 ml.). The reaction may be expressed as:

$$HCN + S \xrightarrow{\text{rhodanese}} HCNS$$

The source of the sulfur is not known. It can be supplied by thiosulfate *in vitro*, but it remains uncertain whether this compound is utilized physiologically.

Conjugation. The isolation by Keller in 1842 of hippuric acid after the ingestion of benzoic acid led to the recognition of a new type of biochemical reaction, namely, the conjugation of a foreign organic compound with a normal metabolic product such as glycine. Other compounds or radicals employed by the organism for conjugation are glucuronic acid, sulfuric acid, cysteine, glutamine, ornithine, acetic acid, and the methyl group.

Antimetabolites. Though it is generally assumed that conjugated products are the resultants of detoxication, it is more likely that they are merely the end products of normal metabolic processes applied to foreign compounds. When any ingested compound is excreted in a conjugated form, it may be regarded as an antimetabolite (see Chapter 36). One may postulate that such a compound has enough structural similarity to a normal metabolic product to engage the enzyme designed for the latter.

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[2] Quick and Sweet: *J. Biol. Chem.*, **80**, 527 (1928).

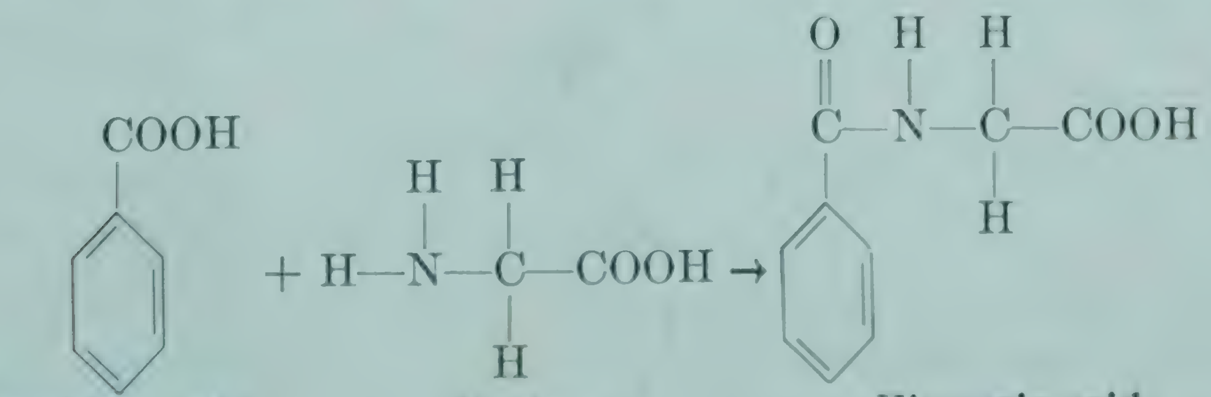
As a result the normal activity of the enzyme is decreased, and it produces an abnormal product which cannot be further metabolized and is therefore excreted.

Valuable information has been gained from the study of those conjugated compounds which were the first historically to serve as metabolic tracers. Knoop, as already stated, postulated the theory of β-oxidation from his observations of the end products of phenylaliphatic acids. Glycine, glutamine, ornithine, sulfuric acid, and glucuronic acid were discovered as constituents of conjugated products long before it was recognized that these compounds participated actively in many normal physiological processes. Methylation of pyridine and nicotinic acid was noted decades before biochemists had any appreciation of the essential role of this reaction in metabolism.

The type of conjugation is determined by the active chemical groups in the molecule, but important modifications are brought about by secondary groups not directly concerned in the conjugation. Marked species differences in conjugation occur.

The Carboxyl Group. The introduction of a carboxyl group into the benzene ring is one of the most effective means of reducing toxicity. Thus the addition of the COOH group to phenol produces salicylic acid, a nontoxic compound. Toluene, which the body oxidizes to benzoic acid, is less toxic than benzene, which yields on oxidation phenol, polyphenols, and muconic acid. Benzoic acid is the type compound in which a carboxyl group is attached to the benzene ring. In man nearly all of the compound is combined with glycine to form hippuric acid. There is good evidence that the synthesis occurs primarily in the liver. The quantitative estimation of the excretion of hippuric acid following the administration of benzoic acid is therefore a satisfactory test of liver function. With this method, the body's capacity to synthesize glycine and to conjugate it with benzoic acid are measured. The organism can utilize waste nitrogen for this synthesis, but the exact reactions involved are not known.

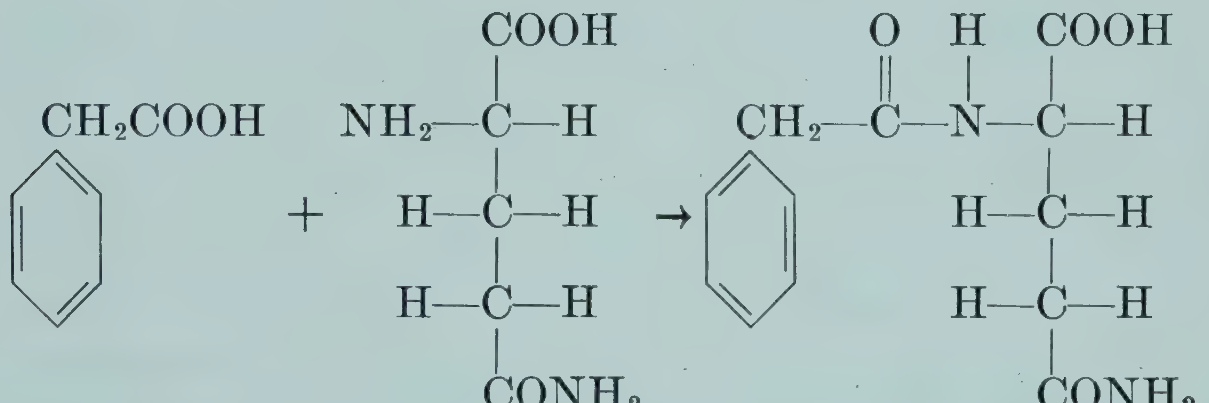
Rather than postulate that the body possesses a mechanism specifically designed to synthesize hippuric acid, it is more reasonable to suppose that benzoic acid has enough structural resemblance to a metabolite normally conjugated with glycine by a particular enzyme system to be acted upon by the same enzyme. As a result, benzoic acid is conjugated with glycine to form hippuric acid, which is excreted because it cannot be further metabolized. During the period that benzoic acid is being conjugated, the enzyme is inhibited from carrying out its normal function. Evidence



of such a metabolic depression is the observation that the excretion of uric acid is strikingly decreased after the ingestion of benzoic acid.

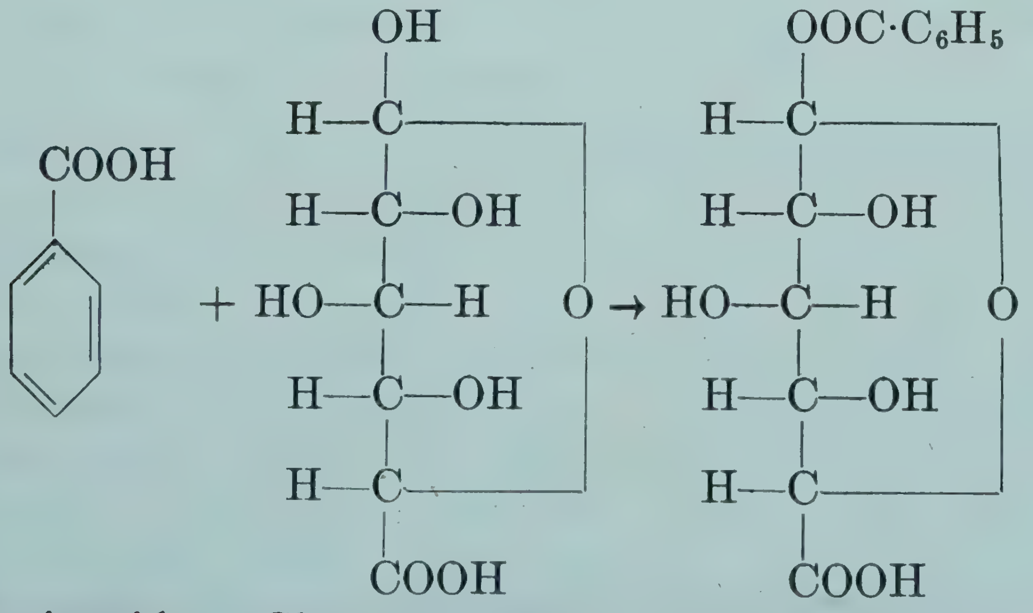
Substitutions in the benzene ring of benzoic acid alter the pattern of conjugation. Any group in ortho position strikingly slows or inhibits the conjugation with glycine. This is well illustrated by the important drug, salicylic acid. Irrespective of the size of the dose, little of the compound is combined with glycine and excreted as salicyluric acid. Interestingly, when the hydroxy group is in para position, man excretes about one-half of the ingested compound unconjugated, and the other half combines with glycine to form *p*-hydroxyhippuric acid. *p*-Methoxybenzoic acid, in marked contrast, is half conjugated with glucuronic acid and the remainder with glycine.

Phenylacetic acid differs sufficiently in structure from benzoic acid to require a different enzyme for its conjugation. This is demonstrated by the fact that when both kidneys are removed from a dog, the animal loses the power to synthesize hippuric acid, but retains the ability to form phenylaceturic acid. It is of considerable interest that phenylacetic acid is the only compound known to be conjugated with glutamine. This synthesis has been observed only in man and in the chimpanzee. In the avian organism both benzoic and phenylacetic acid are conjugated with ornithine.



Phenylacetic acid **Glutamine** **Phenylacetylglutamine**

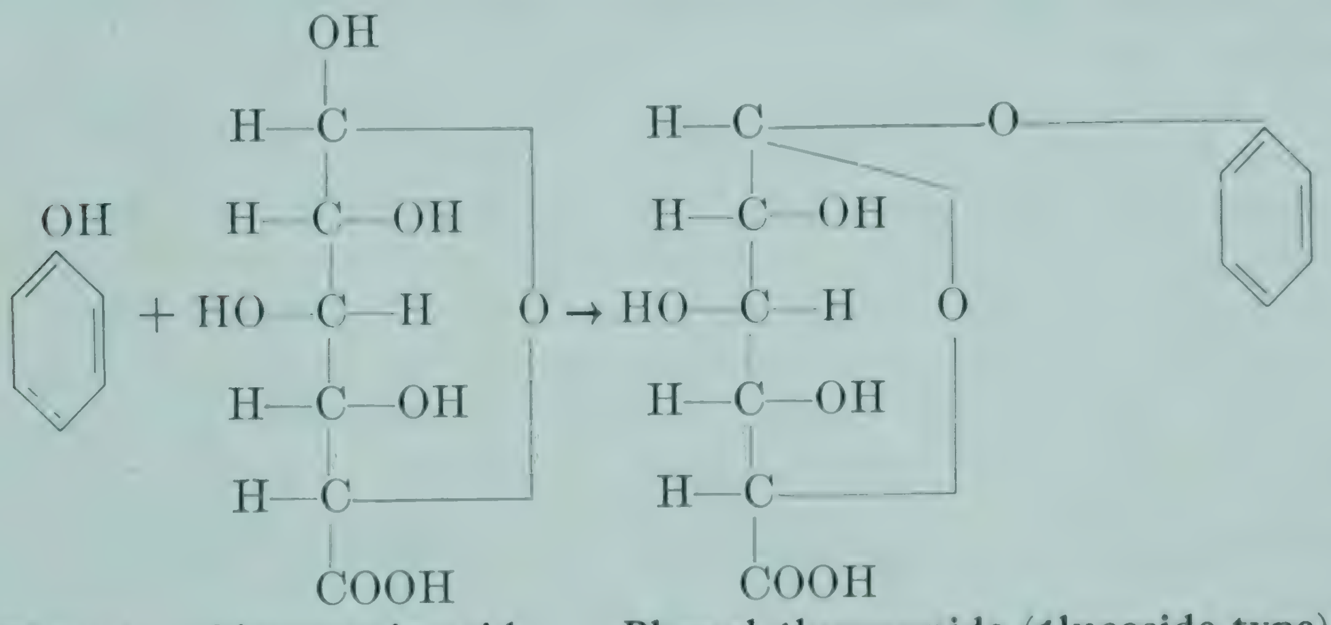
The dog, in contrast to man and the rabbit, conjugates nearly 75 per cent of the ingested benzoic acid with glucuronic acid. The source of this compound remains unknown. Free glucuronic acid is poorly metabolized, and since it is not found in the unconjugated form, it seems unlikely that a



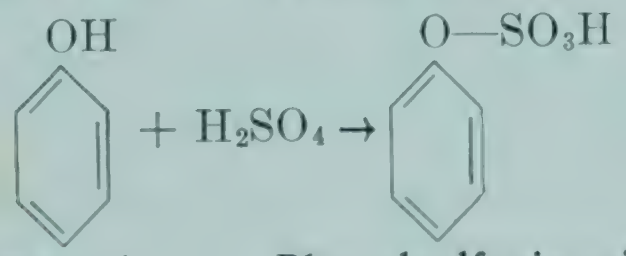
Benzoic acid **Glucuronic acid** **1-Benzoylglucuronic acid**

direct union of glucuronic acid and benzoic acid occurs. The observation that the completely diabetic organism retains the ability to form conjugated glucuronides, apparently from the fraction which would be excreted as glucose, shows that glucogenic amino acids can serve as a source of glucuronic acid. Whether the foreign compound is combined with glucose with subsequent oxidation to glucuronic acid or whether the glucuronic acid is synthesized from three carbon compounds remains unsolved. The wide distribution of the enzyme β-glucuronidase in the body and the observation that various sex hormones are combined and excreted with glucuronic acid clearly indicate that this compound occupies a prominent position in metabolism.

The Hydroxyl Radical. A compound such as phenol is conjugated with both glucuronic acid and sulfuric acid. The latter type of compound is called an ethereal sulfate. Indican, which is the common name for indoxylsulfuric acid, is historically important since it has been used as a measure of intestinal putrefaction. The organism apparently can combine sulfuric acid directly with a phenol. Chloral hydrate ($CCl_3CH(OH)_2$) a commonly employed sedative, is reduced in the body to trichloroethyl alcohol, which is conjugated with glucuronic acid.

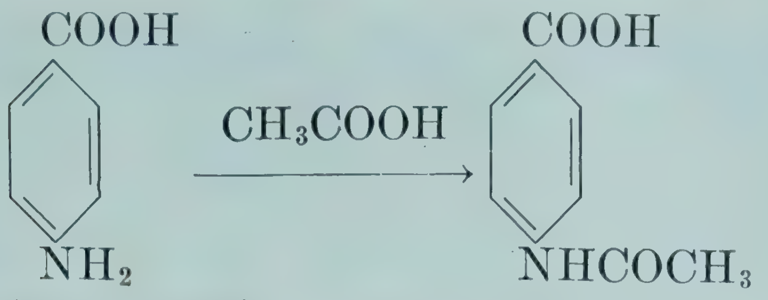


Phenol **Glucuronic acid** **Phenol glucuronide (glucoside type)**

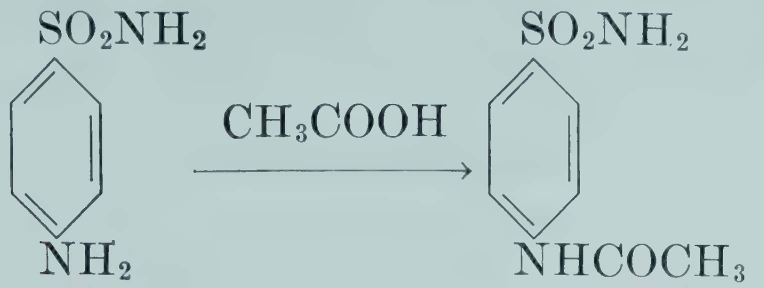


Phenol **Phenylsulfuric acid**

The Aromatic Amino Group. Though aniline is extremely toxic, its acetyl derivative, acetanilide, is a relatively nontoxic drug. It would appear logical therefore to expect acetylation to be an important detoxication reaction. Actually there is little evidence that this is true. Sulfanilamide and its various derivatives are acetylated in the body. Their therapeutic effectiveness is thereby lost, but their toxicity does not appear to be significantly lowered. Interestingly, the vitamin *p*-aminobenzoic acid is acetylated and excreted. Although acetylcholine is an important physiological compound, acetylation of other hydroxy compounds, particularly nonmetabolites, has apparently not been observed.

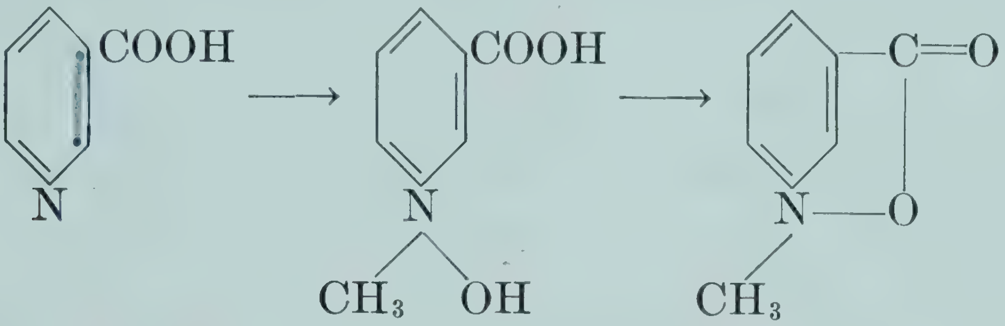


***p*-Aminobenzoic acid** **_p_-Acetylaminobenzoic acid**



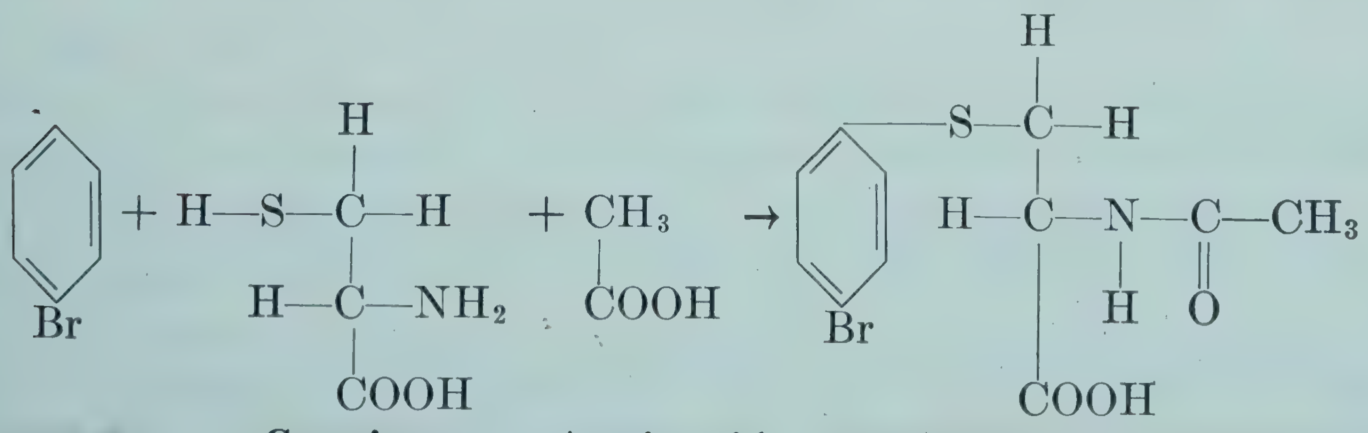
Sulfanilamide (_p_-Aminobenzene sulfonamide) **_p_-Acetylaminobenzene sulfonamide**

The Pyridine Ring. Pyridine, quinoline, and many of their derivatives are methylated in the body. The transfer of a methyl group to nuclear nitrogen is apparently a common reaction. Nicotinic acid is methylated to form trigonelline. Since its antipellagra properties are thereby lost, the purpose of such a reaction is not clear.



Nicotinic acid **Trigonelline**

The Benzene Ring. When benzene or polycyclic hydrocarbons such as naphthalene and anthracene are ingested, their corresponding mercapturic acids can be isolated from the urine. In this conjugation, the sulfur of cysteine becomes directly combined with a nuclear carbon atom, and the amino group becomes acetylated. *p*-Bromophenylmercapturic acid formed from bromobenzene is the best known since it can be isolated fairly easily. Rather than regard this conjugation as a detoxication process, it seems more reasonable to suppose that it serves as a means for introducing hydroxy groups into the benzene ring, which is a step in the metabolism of the aromatic nucleus. This assumes that the body possesses an enzyme which splits the acetylated cysteine from the benzene ring by hydrolysis.



Bromobenzene **Cysteine** **Acetic acid** **Bromophenylmercapturic acid**

The conjugation reactions should be regarded as important metabolic processes rather than biochemical oddities. The ease with which the body furnishes glycine, glucuronic acid, acetic acid, cysteine, and other conjugating components serves as evidence of their wide and important utilization in metabolism. Interestingly, the avian organism lacks the ability to form hippuric acid, but conjugates aromatic acids with ornithine. This suggests the close relationship of some of the conjugating reactions with the Krebs-Henseleit urea cycle.

EXPERIMENTS

INDOLE AND SKATOLE

1. *Herter's β-Naphthoquinone Reaction.* To 10 ml. of unknown solution add 2 drops of 2 per cent solution of β-naphthoquinone sodium monosulfonate and 2 ml. of 10 per cent NaOH. Let stand 15 minutes. Shake with 2 ml. of chloroform. A pinkish-red color in the chloroform indicates indole. This is a very delicate test.

2. *Ehrlich's p-Dimethylaminobenzaldehyde Reaction.* To 10 ml. of unknown solution add 1 ml. of 5 per cent alcoholic solution of *p*-dimethylaminobenzaldehyde and 1 ml. of concentrated HCl. Indole gives a red color and skatole a blue color.

PHENOLS, HYDROXY AROMATIC ACIDS, AND IMIDAZOLES

Conjugation with Glucuronic Acid and Glycine: Principle. The fate of anisic acid (*p*-methoxybenzoic acid) in the body illustrates the influence exerted by a relatively inert chemical group such as the methoxy radical when introduced into the benzene ring of benzoic acid. In the human organism benzoic acid is conjugated almost entirely with glycine to form hippuric acid, whereas only about 50 per cent of *p*-methoxybenzoic acid is combined with glycine; the remainder is conjugated with glucuronic acid. When glycine or gelatin is given with the anisic acid, the amount of *p*-methoxyhippuric acid excreted is greatly increased without, however, depressing the conjugation with glucuronic acid.

***Procedure.* 3 g. of anisic acid is neutralized exactly with 10 per cent sodium hydroxide, using phenolphthalein as the indicator. The solution is diluted to 30 ml. and ingested. 200 ml. of water are drunk. The subject voids immediately after taking the drug, and then collects complete hourly specimens for three hours. Each specimen is measured and its reducing action tested by mixing five drops with 5 ml. of Benedict's qualitative sugar reagent in a test tube and heating. The glucuronic acid can also readily be determined quantitatively by using Benedict's quantitative sugar method. 10 ml. of Benedict's quantitative reagent is transferred to a small Erlenmeyer flask or porcelain evaporation dish and 4 g. of anhydrous sodium carbonate added. The mixture is heated to boiling and the undiluted urine added from a 5-ml. Mohr pipet until the blue color completely disappears. This is a preliminary determination. For more exact results the urine is so diluted that about 10 ml. of the diluted urine is required to reduce 10 ml. of the reagent. The same quantity of Benedict's reagent and sodium carbonate as before is used. The diluted urine is delivered with a buret into the reagent, which is kept boiling. The glucuronic acid equivalent is approximately the same as that of glucose. The glucuronic acid may also be determined photometrically**

by the naphthoresorcinol test.[3] The remainder of the sample is acidified with 1 ml. of concentrated hydrochloric acid and stirred to precipitate *p*-methoxyhippuric acid. Inoculation with a small crystal of the compound will hasten precipitation. The crystalline product is filtered by suction, washed with a small amount of cold water, and dried. From the weight of the product plus the amount calculated that remained in solution (100 ml. of urine dissolve 0.24 g. of *p*-methoxyhippuric acid), the quantity of anisic acid which is conjugated with glycine can be calculated.

QUANTITATIVE BROMINATION

Method of Day and Taggard:*[4] *Principle. Phenol, aniline, and many of their derivatives such as hydroxy- and aminoaromatic acids are readily brominated. A quantitative replacement of one or more nuclear hydrogens by bromine occurs. From the amount of bromine consumed, the quantity of aromatic compound is calculated.

***Procedure.* 0.2 g. of *p*-methoxyhippuric acid obtained in the preceding experiment and purified by recrystallization from hot water is neutralized with sodium hydroxide and diluted to 75 ml. The solution is quantitatively transferred to a 500-ml. glass-stoppered bottle, and 25 ml. of 0.2 N bromate solution (75 g. of KBr and 5.6 g. of $KBrO_3$ per liter) is added. The solution is acidified with 5 ml. of concentrated hydrochloric acid and shaken for one minute. After 30 minutes the reaction bottle is cooled in ice or under the tap. The stopper is dislodged sufficiently to permit adding 5 ml. of 40 per cent KI solution. Care must be taken to prevent bromine vapor from escaping. The liberated iodine is titrated with 0.1 N sodium thiosulfate, using starch solution as indicator. The amount of bromine consumed is calculated from the difference between the number of ml. of thiosulfate required in the titration of the sample and the titration of the blank using 25 ml. of 0.2 N bromate solution.**

CALCULATION. The bromine is supplied by the reaction:

$$KBrO_3 + 5KBr + 6HCl = 3Br_2 + 3H_2O + 6KCl$$

One molecule of *p*-methoxyhippuric acid reacts with one molecule of bromine:

$$CH_3OC_6H_4COOH + Br_2 = CH_3OC_6H_4BrCOOH + HBr$$

Each ml. of 0.1 N thiosulfate is equivalent to 0.01045 g. of *p*-methoxyhippuric acid.

For the bromine equivalent of other amino and phenolic compounds the original articles of Day and Taggard and of Quick should be consulted.

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Stekol: "Detoxication mechanisms," *Ann. Rev. Biochem.,* **10,** 265 (1941).

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[3] Maugham, Evelyn, and Browne: *J. Biol. Chem.,* **126,** 567 (1938); Brag, Humphris, and White: *Biochem. J.,* **52,** 412 (1952).

[4] Day and Taggard: *Ind. and Eng. Chem.,* **20,** 545 (1928); Quick: *J. Biol. Chem.,* **97,** 403 (1932).

21

Feces

General. The feces include the residue remaining in the intestine after the digestion and absorption of food together with products of intestinal secretion, epithelial debris, and bacterial growth and decomposition.

They are composed of the following substances:

1. Food residues, i.e., those portions of the food which either escape or are incapable of digestion and absorption.
2. The remains of the intestinal and digestive secretions not destroyed or reabsorbed.
3. Substances excreted into the intestinal tract, notably phosphates and other salts of calcium, iron, and other metals.
4. The bacterial flora of the intestinal tract and their metabolic end products.
5. Cellular elements to which may be added, under pathological conditions, blood, pus, mucus, serum, and parasites.
6. Abnormally, enteroliths, gallstones, and pancreatic calculi.

The amount of the fecal discharge varies with the individual and the diet. Upon an ordinary mixed diet various authorities claim that the daily excretion by an adult male will aggregate 110–170 g. with a solid content ranging between 25 and 45 g.; the fecal discharge of such an individual upon a vegetable diet will be much greater and may even be as great as 350 g. and possess a solid content of 75 g. In the authors' experience the average daily output of moist feces, calculated on the basis of data secured from the examination of over 1,000 stools, was about 100 g. The variation in the normal daily output is so great that this factor is of very little value for diagnostic purposes, except where the composition of the diet is accurately known. Lesions of the digestive tract, defective absorptive function, or increased peristalsis, as well as an admixture of mucus, pus, blood, and pathological products of the intestinal wall, may

INFLUENCE OF DIET ON FECAL DRY MATTER

	Diet	*Dry Matter (Per Cent)*
Milk	Nursing infant	15.0
	Adult	28.0
Meat		29.0
Bread		25.0
Potatoes		15.0
Cabbage		4.4
Mixed diet		26.0

cause the total amount of excrement to be markedly increased. An idea of the variation of the percentage of dry matter in the feces, evacuated after the ingestion of different diets, may be gathered from a consideration of the preceding table.

Fecal Pigments. The principal pigment of the feces is stercobilin which is chemically identical with the urobilin of urine, and like it is formed by oxidation of a chromogen stercobilinogen (or urobilinogen). This explains the darkening of stools upon exposure to air. The primary precursor of these compounds is bilirubin, which is reduced by intestinal bacteria to mesobilirubinogen; in the presence of a factor from bile this intermediate is transformed to urobilinogen or stercobilinogen, according to the relations shown on p. 413. A rapid decline to negligible amounts of urobilinogen in the feces, bile, and urine has been shown to follow the administration of aureomycin in therapeutic doses. Neither bilirubin nor biliverdin occurs normally in the fecal discharge of adults, although the former may be detected in the excrement of nursing infants. If these pigments are found in the feces of adults, they indicate an abnormally rapid transit through the large bowel, preventing their transformation into stercobilinogen. A simple method has been suggested[1] for the isolation of stercobilin and urobilin from the feces in crystalline form. The color of the fecal discharge is greatly influenced by the diet. A mixed diet, for instance, produces stools which vary in color from light to dark brown, an exclusive meat diet gives rise to a brownish-black stool, whereas the stool resulting from a milk diet is invariably light colored. Drugs and certain pigmented foods, such as cocoa, beets, the chlorophyllic vegetables, and various varieties of berries, each afford stools having a characteristic color. This is well illustrated by the occurrence of yellow stools following the administration of rhubarb, senna, or santonin, and of red stools following prontosil treatment. The green color of the calomel stool is generally believed to be due to biliverdin. The black stools following the administration of bismuth or iron drugs result from the formation of metallic sulfides or suboxides. In cases of biliary obstruction the absence of pigment and the presence of excess fat result in the formation of grayish-white acholic stools. Barium meals used in roentgenographic diagnosis impart a clay-white color to the feces.

Odor. Under normal conditions the odor of feces is due to skatole and indole, two substances formed in the course of putrefactive processes occurring within the intestine (see Chapter 20). Such compounds as methyl mercaptan, hydrogen sulfide, and similar substances resulting from bacterial putrefaction may also add to the disagreeable character of the odor. The intensity of the odor depends to a large degree upon the character of the diet, being very marked in stools from a meat diet, much less marked in stools from a vegetable diet, and frequently hardly detectable in stools from a milk diet. Thus the stool of the infant is ordinarily nearly odorless and tends to be rancid rather than putrefactive; it is believed that any decided odor may generally be readily traced to some pathological source.

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[1] Watson, Lowry, Sborov, Hollinshead, Kohan, and Matte: *J. Biol. Chem.*, **200**, 697 (1953).

Reaction. Experiments in which the actual hydrogen-ion concentration of the feces was determined indicate that the normal reaction of the excreta is slightly alkaline (pH 7.0 to 7.5). Pronounced dietary changes, (e.g., low-protein diet, high-protein diet, fasting, water-drinking with meals) produce at most only minor changes in the reaction of the feces. The ingestion of large amounts of lactose may cause the production of an acid reaction.

Consistency. The form and consistency of the stool is dependent, in large measure, upon the nature of the diet. Under normal conditions the consistency may vary from a thin, pasty discharge to a firmly formed stool. Stools which are exceedingly thin and watery ordinarily have a pathological significance. In general the feces of the carnivorous animals are of a firmer consistency than those of the herbivora.

The continued ingestion of a diet which is very thoroughly digested and absorbed is frequently accompanied by the formation of dry, hard, fecal masses (*scybala*). Constipation generally results from the small bulk of the feces and its lack of moisture. At present the formation of scybala is considered pathological, as an expression of spastic constipation. To counteract this tendency toward constipation the ingestion of agar-agar, psyllium seed, or other vegetable gums is practiced. These are relatively indigestible and readily absorb water, thus forming a bulky fecal mass which is sufficiently soft to permit easy evacuation. Mineral oil, because of its lubricating and softening properties is much used as an aid in overcoming constipation. Though it is inert and practically nonabsorbable, excessive amounts of mineral oil may interfere with the absorption of fat-soluble vitamins.

Separation. It is frequently desirable for clinical or experimental purposes to make an examination of the fecal output which constitutes the residual mass from a certain definite diet. Under such conditions, it is customary to cause the person under observation to ingest at the beginning and end of the period in question some substance sufficiently different in color and consistency from the surrounding feces to render comparatively easy the differentiation of the feces of that period from the feces of the immediately preceding and succeeding periods. One of the most satisfactory methods of making this separation is by means of the ingestion of a gelatin capsule containing about 0.2 g. of powdered charcoal at the beginning and end of the period under observation. This procedure causes the appearance of two black zones of charcoal in the fecal mass. A capsule containing carmine (0.3 g.) may be used in a similar manner and forms two dark red zones. Chromic oxide (Cr_2O_3) has also been suggested for this purpose.[2] Some similar method for the separation of feces is usually practiced in connection with accurate nutrition or metabolism experiments conducted for the collection of useful data regarding the income and output of nitrogen and other elements.

Macroscopical Examination. Among the macroscopical constituents of the feces may be mentioned the following: Intestinal parasites and their ova, undigested food particles, gallstones, pathological products of the intestinal wall, enteroliths, intestinal sand, and objects which have been accidentally swallowed.

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[2] Lloyd and Crampton: *J. Nutrition*, **41**, 629 (1950); Irwin and Crampton: *Ibid.*, **43**, 77 (1951).

Microscopical Examination. The fecal constituents which at various times and under different conditions may be detected by the use of the microscope are as follows: (1) Constituents derived from the food, such as muscle fibers, connective-tissue shreds, starch granules, and fat; (2) formed elements derived from the intestinal tract, such as epithelium, erythrocytes, and leukocytes; (3) mucus; (4) pus corpuscles; (5) parasites; and (6) bacteria. In addition to the constituents named, the following crystalline deposits occasionally may be detected: cholesterol, coprosterol, soaps, fatty acid, fat, hematoidin, "triple phosphate," Charcot-Leyden crystals (see Fig. 104), and the oxalate, carbonate, phosphate, sulfate, and lactate of calcium.

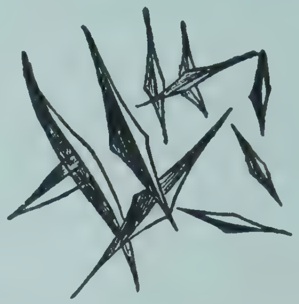
FIG. 104. CHARCOT-LEYDEN CRYSTALS.

Fat in Feces. The amount and composition of fat excreted by way of the feces is largely independent of food fat but tends to approximate the composition of blood lipides. Fecal fat is largely of endogenous origin, the secreted fat being partially reabsorbed; only to a minor degree does fecal fat come from epithelial debris and bacteria.

About one-third of the fat in normal feces is unsaponifiable. The coprosterol (also called coprostanol) of the feces is similar to cholesterol, and is formed by the reduction of the latter. It contains two more atoms of hydrogen than cholesterol and is thus a saturated alcohol $C_{27}H_{47}OH$. A small amount of cholestanol, an isomer of coprosterol, is also found in feces. It appears to be formed in the tissues and excreted in the bile. Except for the D vitamins, cholesterol is the only sterol absorbed from the intestine. The phytosterols of plants are not absorbed, being excreted in the feces. Coprosterol responds to cholesterol color tests and has the same solubility, but possesses a lower melting point and crystallizes as fine needles instead of plates such as cholesterol forms.

After the intravenous administration of cholesterol–4–C^{14} to normal rats it was found[3] that from 10 to 14 per cent of the total C^{14} in bile and from 17 to 23 per cent of fecal C^{14} were recovered in the nonsaponifiable fraction. This includes cholesterol, dihydrocholesterol, coprosterol, etc. The fatty acid fraction contained little if any C^{14}. About 80 per cent of the fecal C^{14} and about 90 per cent of the bile C^{14} was present in the form of bile acids.

The fat content of normal feces may vary from 5 to 25 per cent (on a dry basis). Excessive excretion of fat via feces is known as steatorrhea, a condition due to failure to absorb fat as in enteritis, hepatic disorder, biliary obstruction, celiac disease, or sprue. Excreted fat is partly saponified and may entail loss of calcium, as soap, to the extent of producing pathological demineralization.

Blood in Feces. The detection of minute quantities of blood in the feces—so-called occult blood—is an aid to the correct diagnosis of certain disorders. In these instances the hemorrhage is ordinarily so slight that the identification by means of macroscopical characteristics as well as the microscopical identification through the detection of erythrocytes are both unsatisfactory in their results. Of the tests given for the detection of occult blood the benzidine reaction is probably the most satisfactory.[4] Since occult blood occurs with considerable regularity and frequency in gastrointestinal cancer and in gastric and duodenal ulcer, its detection in the feces is of especial value as an aid to a correct diagnosis of these disorders. Certain precautions are essential, such as the establishment of a meat-free diet over a period of time before the specimen is collected. (Feces from a meat diet will give an occult blood reaction with some of the most delicate tests.) Bleeding from the bowel such as is seen in hemorrhoids, as well as the admixture of menstrual blood, is to be considered in the interpretation of the result. After the ingestion of 50 ml. of human blood 85 to 95 per cent of the hemoglobin appears as protohemin in the feces.

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[3] Siperstein, Jayko, Chaikoff and Dauben: *Proc. Soc. Exptl. Biol. Med.*, **81**, 720 (1952).

Bacteria in Feces. It has been quite clearly shown that the intestine of the newly born is sterile. However, this condition is quickly altered, and bacteria may be present in the feces before or after the first ingestion of food. There are three possible means of infecting the intestine; i.e., by way of the mouth or anus or through the blood. The infection by means of the blood seldom occurs except under pathological conditions; thus the usual sources are limited to the mouth and anus.

In infants with pronounced constipation, two-thirds of the dry substance of the stools has been found to consist of bacteria. In the stools of normal adults probably about one-third of the dry substance is bacteria. The average excretion of dry bacteria in 24 hours for an adult is about 8 g. The output of fecal bacteria has been found to undergo a decrease under the influence of water-drinking with meals. There is also a decrease in intestinal putrefaction, a fact which indicates that at least a part of the bacterial deficit is made up of putrefactive organisms. In some cases, more than 50 per cent of the total nitrogen of feces has been shown to be bacterial nitrogen.

Some of the more important organisms met with in the feces are the following: *E. coli*, *B. lactis aerogenes*, *Cl. welchii*, *B. bifidus*, and *coccal* forms. Of these the first three types mentioned are gas-forming organisms. The production of gas by the fecal flora in dextrose bouillon is subject to great variations under pathological conditions; alterations in the diet of normal persons will also cause wide fluctuations. Data as to the production of gas are of considerable importance in a diagnostic way, although the exact cause of the variation is not yet established. It should be borne in mind in this connection that gas volumes are frequently variable with the same individual. For this reason it is necessary in every instance to follow the gas production for a considerable period of time before drawing conclusions.

After aureomycin therapy, coliform organisms entirely disappear from the feces, while the clostridia diminish in quantity and may even disappear. No appreciable influence on the fecal flora has been shown to follow the parenteral administration of penicillin and dihydrostreptomycin in combination.

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[4] **Hughes: *Brit. Med. J.*, 2, 970 (1952).**

Enzymes in Feces. Various enzymes have been detected in the feces. The first one so demonstrated was pancreatic amylase. The amylase content of the feces has been considered to be an index of pancreatic activity. The excretion of this enzyme has been found to increase under the influence of water-drinking with meals. Other enzymes which have been found in the feces under various conditions are trypsin, rennin, maltase, sucrase, lactase, nuclease, and lipase. In an abnormally rapid transit of food through the intestinal tract, such as is seen in certain diarrheas, nearly all of these enzymes may be detected.

Vitamins in Feces. A variety of vitamins are present in normal feces. Their number and amount are subject to wide variation under pathological conditions. For example, the feces of enteritis patients have been found to be deficient in vitamins B_1 and B_6 (but not B_2) compared with feces of normal persons.[5] Pennington[6] reports a heat-labile vitamin B_{12} complex in feces of the rat.

Fecal Nitrogen. The nitrogen present in the feces exists principally in the form of (1) bacteria, (2) unabsorbed intestinal secretions and digestive juices, (3) epithelial cells, (4) mucous material, (5) food residues, and (6) amino acids. In the early days of nutrition study the fecal nitrogen was believed to consist principally of food residues. We now know that such residues ordinarily make up but a small part of the total nitrogen of the stools of normal individuals who exercise normal mastication. When meat has been bolted, however, from 0.5 to 16 g. of macroscopical meat residues have been found in a single stool. The phrase "metabolic-product nitrogen" has been used as a designation for all fecal nitrogen except that present as food residues and bacteria. Bacteria cannot logically be classed under metabolic nitrogen since they doubtless develop at the expense of food nitrogen as well as at the expense of that in the intestinal secretions. In the accurate study of protein utilization[7] a correction should be made for *metabolic nitrogen.* Data regarding the output of metabolic nitrogen may be secured by determining the fecal-nitrogen excretion on a diet of proper energy value but *containing no nitrogen.* However, to prevent tissue catabolism from exceeding its normal level, it is customary in studies of basal nitrogen metabolism to include a minimal percentage of adequate protein in the maintenance diet.

Amino Acids in Feces. Fecal amino acids are derived from protein digestion as well as from intestinal organisms and secretions. It has been shown[8] that excretion of free amino acids was more abundant in the feces of the breast-fed infant than in those of the artificially fed infant. The feces of the breast-fed infant showed alanine to be the predominant amino acid, whereas in the feces of the artificially fed baby valine and lysine predominated. In experiments on white rats it has been shown that fecal excretion of lysine, valine, histidine, and methionine is much higher on diets supplying potato starch (either raw or autoclaved) or dextrin than on those containing sucrose or glucose.[9]

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[5] Diaz *et al.: Rev. clín. españ.*, **44,** 233 (1952).

[6] Pennington: *Biochem. J.*, **48,** xviii (1951).

[7] See Protein Utilization in Chapter 33.

[8] Ross: *Lancet,* **241,** 190 (1951).

A method has been suggested for the determination of metabolic fecal phosphorus[10] excretion through the use of casein labeled with P^{32}.

Fasting Feces. Feces are still excreted from the intestine even when no food is ingested. Carefully conducted fasting experiments have demonstrated this. A dog nourished on an ordinary diet to which bone ash has been added will excrete gray feces. When fasted, such an animal will after a few days excrete a small amount of a greenish-brown mass, containing no bone ash. These are fasting feces. They are of a pitchlike consistency and turn black on contact with the air. Adult fasting men have been found to excrete 7 to 8 g. of feces per day, the daily nitrogen value being about 0.1 g. No separating medium such as charcoal or carmine (p. 448) should be used in differentiating fasting feces.

Examination for Clinical Purposes. The examination of feces for evidences of parasitism (detection of parasites and their ova), such as hookworm, tapeworm, etc., is of considerable clinical importance. For diagnostic purposes the macroscopical and microscopical examinations of the feces ordinarily yield much more satisfactory data than are secured from its chemical examination. Possibly with the exception of certain examinations for occult blood, the most satisfactory data for diagnostic purposes are secured by microscopical examination. This presupposes a knowledge of microscopical technique and the use of certain microchemical tests, by which much information can be obtained. The principle underlying this examination consists in the study of the actual changes which the various foodstuffs have undergone during digestion. A knowledge of the changes which occur in normal digestion and which are seen in normal feces enables one readily to detect pathological variations. One diet widely used for this purpose is the Schmidt diet. The modification[11] described below is better adapted to American conditions.

Modified Schmidt Diet

Breakfast:
- 100 g. of farina or oatmeal
- 60 g. of toast
- 20 g. of butter
- 250 ml. of milk

Luncheon:
- Rice soup (chicken broth with rice)
- 100 g. of green vegetable (asparagus)
- 100 g. of mashed potato
- 60 g. of toast
- 20 g. of butter
- 250 ml. of milk

4 o'clock:
- 250 ml. of milk

Dinner:
- 150 g. of chopped meat, grilled on the outside and rare in the center
- 100 g. of green vegetable (spinach)
- 100 g. of mashed potato
- 60 g. of toast
- 20 g. of butter
- 250 ml. of milk
- Stewed fruit

EXPERIMENTS ON FECES

***1. Collection and Preservation of Feces** (see also Chapter 33).* **Friction-top tin cans may be prepared for the collection of stools by adding a few drops of**

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[9] Harper, Katayama, and Jelinek: *Can. J. Med. Sci.*, **30**, 578 (1952).

[10] Lofgreen, Kleiber, and Quick: *J. Nutrition*, **47**, 571 (1952).

[11] Used by Dr. Martin E. Rehfuss of Philadelphia.

formalin or alcoholic solution of thymol (from which the alcohol may be evaporated by warming). Stools may be dried directly in these cans. If glass containers are preferred, pyrex pots with glass lids may be used. To prevent loss of nitrogen sufficient sulfuric acid should be added to render the fecal mixture strongly acid. The contents may be dried on a steam bath in a hood and the residue weighed and powdered for sampling. If nitrogen or mineral analyses are to be conducted, the fresh stools may be mixed with 100 to 200 ml. of water and an equal volume of concentrated H_2SO_4 gradually added while stirring. The homogenized suspension thus collected and preserved throughout the 24 hours may be diluted to volume and an aliquot taken for analysis.

2. *Macroscopical Examination.* If the stool is watery pour it into a shallow dish and examine directly. If it is firm or pasty it should be treated with water and carefully stirred before the examination for macroscopical constituents is attempted. The macroscopical constituents may be collected very satisfactorily by means of a double layer of cheesecloth or a sieve of fine mesh.

3. *Reaction.* Thoroughly mix the feces and apply moist indicator papers to the surface. If the stool is hard it should be mixed with water before the reaction is taken. Examine the stool as soon after defecation as is convenient, since the reaction may change very rapidly. The reaction of the normal stools of adult man is ordinarily neutral or faintly alkaline to litmus, but seldom acid. Infants' stools are generally acid in reaction. The glass electrode is useful in determining fecal pH since it is not affected by reducing substances commonly present, nor by the presence of solids.

4. *Starch.* If any imperfectly cooked starch-containing food has been ingested, it will be possible to detect starch granules by a microscopical examination of the feces. If the granules are not detected by a microscopical examination, the feces should be placed in an evaporating dish or casserole and boiled with water for a few minutes. Filter and test the filtrate by the iodine test in the usual way (see p. 84).

5. ***Blood.*** Undecomposed blood may be detected macroscopically. If uncertain, look for erythrocytes under the microscope, and spectroscopically for the spectrum of oxyhemoglobin (see p. 492).

In case the blood has been altered or is present in minute amount (occult blood) and cannot be detected by the means just mentioned, the following tests may be tried:

BENZIDINE REACTION. (a) Make a thin fecal suspension using about 5 ml. of distilled water. Shake with 5 ml. of ether to remove the fat and discard this ether extract. Acidify the residue with acetic acid and extract again with 5 ml. of ether. Pour the acid ether extract into a small evaporating dish. Evaporate to dryness on a hot water bath (with flame turned out). Add a few drops of water, a drop of saturated solution of benzidine in glacial acetic acid and a drop of 3 per cent hydrogen peroxide. A blue or green color indicates the presence of blood.

(b) SLIDE MODIFICATION. Take up a little of the solid stool on a match, smear it on an object glass, and pour the reagent over it. If there is blood present the smear turns blue and there is no misleading green tint from fluid. Make the solution as follows: Add a knife-tip of benzidine to 2 ml. of glacial acetic acid, and add 20 drops of a 3 per cent solution of hydrogen peroxide.

By this dry technique there is no danger of soiling the fingers, and the test is more sensitive than the usual wet benzidine test. The smear of stool is either blue or it is not blue. The rapidity of the color change gives some idea as to the proportion of blood in the stool; with much blood present the change to blue is instantaneous.

(e) LEVIN-WATT MODIFICATION.[12] **Emulsify a small portion of feces in distilled water. Filter into a clean test tube, using a Whatman No. 5 or a more retentive filter paper. Treat 3 ml. of the filtrate with 8 drops of 50 per cent acetic acid or glacial acetic acid, and mix. Add 8 drops of hydrogen peroxide (C.P. 3 per cent) to the mixture and shake. Make a contact ring by carefully overlaying final mixture drop by drop with an alcoholic benzidine solution.[13] Tilt the test tube in order to bring about slight mixing at the ring. A green color at the area of contact indicates a positive reaction. The intensity of color varies with the concentration of blood.**

6. *Quantitative Determination of Fat in Feces: Principle.* The determination of fat in dried feces is a more or less tedious process, and one which is somewhat inaccurate if applied to pathological feces. Most of the methods for the determination of fat in the moist feces are accurate, but require a long time. Saxon has proposed a method for the determination of fat in moist feces, which is speedy, convenient, and accurate. The soaps of the feces are converted into free fatty acids by means of hydrochloric acid, and the material is then extracted by shaking with ether. The ether removes the neutral fat, the fatty acids which were present as such, the fatty acids derived from the soaps, and the cholesterol. The ether is removed by distillation, the crude fat purified by means of petroleum ether, and the weight of the total fat obtained. The fat is then dissolved in benzene and titrated with tenth-normal sodium alcoholate solution, using phenolphthalein as an indicator. The fatty acid is calculated, from the titration, as stearic acid.

Procedure. **Place about 5 g. (accurately weighed) of the thoroughly mixed feces in a 100-ml. glass-stoppered graduated cylinder.[14]**

Add 20 ml. of distilled water, 1 to 2.5 ml. of concentrated hydrochloric acid (depending upon the amount of the sample), and, again, sufficient distilled water to make a total volume of 30 ml. Add exactly 20 ml. of ether, stopper, and shake vigorously for five minutes. Allow to stand for a few seconds, remove the stopper, add exactly 20 ml. of 95 per cent alcohol, and again shake for five minutes.

Set the cylinder aside. The ether, containing practically all of the fat, will come to the top as a colored transparent layer. Draw the ether layer off into a tall 150- to 200-ml. beaker. The thin layer of ether which remains is diluted with 5 ml. of ether, the tube slightly agitated, and the ether drawn off. This is done in all five times, care being taken each time to wash down the sides of the cylinder. The stopper should also be washed. 20 ml. of ether are

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[12] Levin and Watt: *Rev. Gastroenterol.*, **16**, 650 (1949).

[13] Dissolve 6 g. of benzidine in 100 ml. of 95 per cent ethyl alcohol. It is preferable to use a solution which is 2 to 3 days old.

[14] Care must be taken not to smear the neck of the cylinder. This may be avoided by removing the feces from the weighing bottle by means of a glass rod, the end of which is flattened and bent in the shape of a hoe, and transferring small bits of the feces from the hoe to the cylinder on short pieces of glass rod which are dropped into the cylinder together with the feces. Tests made in the senior author's laboratory have shown that the fat of feces decreases on standing even in the frozen condition. Analyses should be made on fresh feces.

again added, and the cylinder shaken for five minutes and set aside. When the ether has nearly stratified, draw it off and wash as before. During the second washing, stratification will complete itself. Evaporate the ether[15] until no trace of the alcohol which has been carried over with it remains. To the residue add 30 ml. of low-boiling petroleum ether (it should boil below 60° C.), and allow to stand over night. Petroleum ether for this work should be frequently tested for a residue on evaporation. If a residue is left, the ether should be redistilled. Filter the petroleum ether solution of the fat, catch the filtrate and washings in a tall, weighed, 100-ml. beaker, evaporate off the solvent, dry at 90° C., desiccate, and weigh. After weighing, dissolve the contents of the beaker in 50 ml. of benzol, heat almost to the boiling point, add 2 drops of a 0.5 per cent solution of phenolphthalein, and titrate with a decinormal solution of sodium alcoholate.[16]

CALCULATIONS. The weight of total fat is obtained by subtracting the weight of the empty beaker from the weight of the beaker plus the dried fat. The weight of fatty acids (in terms of mg. of stearic acid) is obtained by multiplying the number of ml. of decinormal sodium alcoholate solution by the factor 28.4. The difference between the weight of total fat and the weight of fatty acids is the weight of neutral fat in the sample extracted.

A separate determination without the addition of hydrochloric acid may be run upon the sample, for the purpose of determining the weight of neutral fat and free fatty acids. The difference between this weight and the weight of total fat is the weight of fatty acid present in the original sample in the form of soaps.

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[15] Erlenmeyer flasks of about 200 ml. capacity may be used, instead of beakers, for th collection of the ether drawn from the cylinders. The ether may then be distilled and recovered. The same procedure may be followed in removing the petroleum ether.

[16] See Appendix.

22

Blood, Lymph, and Cerebrospinal Fluid

General. Blood serves as the principal transport system of the body, the heart furnishing the propulsive force. The most important quantitative functions of the blood are to bring oxygen and nutrients to the tissues and to carry away the waste products and deliver them to the excretory organs—the kidneys, lungs, biliary system of the liver, intestinal mucosa, and skin. Blood also plays an important role in coordinating the activities of various tissues through distribution of hormones, in maintaining the pH and the oxidation-reduction potential within narrow limits, in controlling temperature, osmotic pressure, and ionic concentration, in supplying defense against infection, and in guarding against hemorrhage.

The blood makes up about 5 to 7 per cent of the body weight in man, or approximately 2 to 3 liters per sq. m. of body surface, being more nearly proportional to the latter than to weight. Lower relative volumes of blood are observed in obese than in thin individuals. The volume of the circulating blood in a normal individual is maintained within rather narrow limits; any marked change in blood volume, as by sudden severe hemorrhage, etc., has serious consequences and may even result in death. Blood volume may be measured by the addition to the blood of a known amount of a readily detectable and nondiffusible substance, such as certain dyes, hemoglobin derivatives, and radioactive compounds; the extent to which the substance is diluted by the blood is a measure of the total blood volume.

Composition of Blood. Since blood has numerous functions, its composition is necessarily complex. The general composition is summarized in the following table:

I. Cellular fraction
   Volume: 45 per cent
   Constituent cells
      1. Erythrocytes (5,000,000 per cmm.)
      2. Leukocytes (6,000 per cmm.)
      3. Platelets (250,000 per cmm.)
II. Plasma fraction
   Volume: 55 per cent
   A. Nondiffusible constituents
      1. Albumins
      2. Globulins
      3. Fibrinogen
      4. Enzymes, lipides, etc.

B. Diffusible constituents
    1. Catabolic products: urea, creatinine, uric acid, etc.
    2. Anabolic constituents: glucose, amino acids, creatine, etc.
    3. Electrolytes: Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, HCO_3^-, $HPO_4^=$, $H_2PO_4^-$, etc.
    4. Hormones, vitamins, etc.

The specific gravity of whole blood is about 1.060; that of plasma is approximately 1.026. Since the cells are heavier, they can be separated from plasma by centrifugation. The pH averages 7.4; the viscosity 1.7 to 2 (water = 1), the freezing point −0.56° C. and the total osmotic pressure at 37° C. is 7.6 atmospheres.

Blood Plasma and Serum. When plasma is obtained without the addition of an anticoagulant, it is called *native*. Such a plasma, if kept from having contact with a wettable surface and covered with mineral oil to delay the escape of carbon dioxide, retains fairly closely its *in vivo* state and composition. For the usual chemical analysis, plasma obtained by the addition of sodium oxalate to prevent clotting is generally suitable and is commonly employed.

Serum is the fluid obtained when blood clots. It is similar to plasma in composition but lacks fibrinogen and has a diminished concentration of other clotting factors such as prothrombin. Serum is preferable to plasma for the determination of calcium, albumin, and globulin.

The plasma contains about 91 to 92 per cent of water and about 8 to 9 per cent of solid matter, of which about 7 per cent, or more than 85 per cent of the total solids, is protein.

Plasma Proteins. The plasma proteins represent a complex mixture containing a number of components which differ in properties and function. The major component proteins of plasma include (1) fibrinogen, (2) the various globulins, and (3) the albumins; (4) nucleoprotein and (5) seromucoid are also present in limited amounts.

FIBRINOGEN. The fibrinogen of normal human plasma is present in a concentration of about 0.19 to 0.33 g. per 100 ml. of plasma. The average in the Red Cross series was 0.28 g. per 100 ml. Fibrinogen is likewise found in lymph and chyle as well as in certain exudates and transudates. Human blood fibrinogen is said to have a molecular weight of approximately 500,000, although research suggests that it may be as low as 350,000. The molecule is highly elongated, with a length estimated to be from 600 to 700 Ångstrom units and with an axial ratio of about 20 to 1. The amino acid composition is given in Chapter 4. Fibrinogen is insoluble in salt-free water but is soluble in dilute salt solutions. It is the most readily precipitable of all the common blood proteins by concentrated salt solutions, being precipitated upon half-saturation with sodium chloride or 20 per cent saturation with ammonium sulfate. The coagulation temperature of fibrinogen is about 55° C. in neutral solutions.

Fibrinogen is unique among the blood proteins in that it is readily converted into insoluble fibrin by the action of the enzyme thrombin. Research has contributed to the elucidation of the mechanism of this reaction.[1] Apparently when thrombin acts on fibrinogen, a fibrino-peptide is split off and the remaining molecule becomes activated. It polymerizes to form fibrin, which separates as simple and compound fibers which interestingly exhibit cross-striations with a constant periodicity.[2] The fibrin fibers form a meshwork or reticulum which constitutes the framework of the clot. On standing, the clot shrinks, thereby expressing clear serum. This phenomenon, which is called clot retraction, depends on the presence of intact platelets which adhere to the fibrin strands and are in some way responsible for condensing the fibrin mass.

ALBUMINS AND GLOBULINS. The bulk of the plasma proteins consists of the albumin and globulin fractions. The albumin fraction ordinarily preponderates, comprising well over one-half of the total protein of normal human plasma, but this relation may be altered or even reversed in disease. As a class the plasma albumins differ from the globulins in having a greater solubility, a lower molecular weight, and a more acid isoelectric point. Human serum albumin, for example, has a molecular weight of 69,000 and an isoelectric point at pH 5.4, while the γ-globulin of serum (see p. 463) has a molecular weight of 156,000 and an isoelectric point at pH 6.5. Marked differences in amino acid composition between albumins and globulins have been established, as is evident from the data of the table on p. 122 in Chapter 4, but these differences, while significant from an analytical point of view, as yet have not been related to differences in either structure or function.

The albumin fraction of the plasma is relatively homogeneous and well characterized; a number of serum albumins from various sources have been obtained in crystalline form. Some of these crystalline proteins appear to contain carbohydrate as an integral portion of the molecule.

The globulin fraction on the other hand appears to consist of a variety of proteins of somewhat similar general characteristics, but which by suitable means may be further fractionated into a number of components. Although relatively homogeneous fractions of the plasma globulins have been obtained, none of these proteins has as yet been crystallized. From the point of view of solubility, two general types of globulin are recognized, *euglobulin* and *pseudoglobulin*.

Euglobulin is a true globulin in that it is insoluble in salt-free water; pseudoglobulin, while possessing the general properties of the globulins, is soluble in salt-free water. This distinction between two types of globulins, while useful, is by no means well defined. The extensive studies of Sørensen on this subject, already referred to in Chapter 5, have led him to postulate that the globulin fraction of serum represents a loose combination of euglobulin and pseudoglobulin of the type E_pP_q, in which E and P represent euglobulin and pseudoglobulin complexes respectively, combined in the relative proportions of p and q. Fractionation by various means results in a shift in the proportions of E and P with the resultant formation of more soluble and less soluble complexes. Sørensen was unable to prepare a sample of either euglobulin or pseudoglobulin which was entirely free of the other protein. Cohn, McMeekin, *et al.*, have likewise shown[3] that the euglobulin fraction of serum protein (i.e., globulin precipitated by dialysis) is considerably increased in amount over that first obtained by ammonium sulfate fractionation if the precipitated protein is freed from the last traces of salt by electrodialysis and if the solubility is not influenced by the presence of other proteins.

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[1] Lorand: *Nature*, **167**, 992 (1951); Laskowski, Rakowitz, and Sheraga: *J. Am. Chem. Soc.*, **74**, 28 (1952).

[2] Hawn and Porter: *J. Exp. Med.*, **86**, 285 (1947).

Fractionation of the Plasma Proteins. The separation and characterization of the individual protein components of the plasma is of considerable importance. It facilitates study of the chemical nature and physiological function of each protein and of the significance of variation in the protein composition of the plasma in health and disease. It likewise leads to the possibility of the commercial preparation, from human or animal plasma, of purified preparations of the individual proteins for laboratory, clinical, and industrial use.

Methods for separating the plasma proteins from one another are based almost entirely upon (1) differences in physical properties, such as solubility in water, concentrated salt solutions, and other solvents; (2) rate of sedimentation in the ultracentrifuge; and (3) rate of electrophoretic migration. The various plasma proteins do not differ sufficiently in chemical composition or behavior to permit their separation at the present time on a purely chemical basis. Immunological means of separation—i.e., the use of precipitins (cf. Chapter 5)—specific for the individual protein, have certain disadvantages and have been little used.

DIFFERENCES IN PHYSICAL PROPERTIES. The type of fractionation obtained by differences in solubility in water and in concentrated salt solutions is illustrated in the following table which has been adapted from the work of various investigators.[4] The salts listed in the table, ammonium sulfate and sodium sulfate, are those most commonly employed; sodium chloride, magnesium sulfate, and sodium or potassium phosphate may also be used under suitable conditions. It will be seen

FRACTIONATION OF THE PLASMA PROTEINS BY CONCENTRATED SALT SOLUTIONS

Protein	*Solubility in Dist.* H_2O	*Precipitated by*		*Approximate Normal Conc. in g. per 100 ml. Plasma*	*Per Cent of Total Protein*
		Per Cent Saturation with NH_4 *Sulfate*	*G. Sodium Sulfate per 100 ml., at 37° C.*		
Fibrinogen	−	20	..	0.3	4
Euglobulin	−	33	13.5	0.2	3
Pseudoglobulin I	+	40	17.5	1.3	17
Pseudoglobulin II	+	46	21.5	0.5	7
Albumin	+	>50	..	5.2	69

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[3] Cohn, McMeekin, Oncley, Newell, and Hughes: *J. Am. Chem. Soc.*, **62**, 3386 (1940).

[4] Howe: *J. Biol. Chem.*, **49**, 109 (1921); Gutman, Moore, Gutman, McClellan, and Kabat: *J. Clin. Invest.*, **20**, 765 (1941); and others.

that as the concentration of salt is progressively increased, the various fractions become insoluble and will precipitate from solution. Fibrinogen, which is sometimes classified as a globulin because of its solubility characteristics, is the least soluble protein, followed by euglobulin, the pseudoglobulins, and albumin. It is usually considered that *all* the globulins of plasma are precipitated upon half-saturation with ammonium sulfate, or by a 22 per cent concentration of sodium sulfate at 37° C., and that the protein remaining in solution is albumin. This is the common analytical basis for separation of the albumin and globulin fractions of plasma prior to their analytical estimation.

The fractionation represented in the table above must be regarded as being quite arbitrary. The precipitation limits are not sharply defined, as might be inferred from the table, but rather represent arbitrarily established limits which in reality correspond to zones, between which there is no well-defined transition. The quantitative values given in the table are therefore characteristic of this particular type of fractionation only, and should not be expected to agree with values obtained by fractionation by other methods. Despite this limitation, the method has proved of value in many studies on the variation in plasma protein fractions in health and disease.

Fractionation of the plasma proteins by various concentrations of aqueous alcohol is described by Cohn, Luetscher, *et al.*[5] While alcohol denatures proteins readily at room temperature, denaturation does not occur at temperature ranges of 0° to −5° C., and satisfactory fractionation of the general nature of that obtained by the use of concentrated salt solutions is obtained. The advantage of alcohol fractionation appears to lie chiefly in the ease with which the fractionating agent may be removed from the protein, along with water, in the process of preparing stable dried preparations of the various fractions (lyophilization), thus rendering the method applicable to the large-scale preparation of such dry protein fractions for clinical and industrial use.

By means of varying the temperature, the alcohol concentration, and the pH, Cohn and associate were able to separate the plasma proteins into six fractions which they designated by Roman numerals. Thus Fraction I, which contains most of the fibrinogen as well as a high concentration of the factor which is deficient in hemophilia, is obtained at −3° C. with 8 to 10 per cent alcohol at pH 7.2. To obtain the other fractions, the alcoholic concentration is progressively increased and the pH is lowered. Of particular clinical importance is Fraction V which contains nearly all of the albumin. None of the fractions, however, contain pure components. Most of the γ-globulin is distributed between Fractions II and III. This globulin furnishes definite protection against the paralytic effects of poliomyelitis.

The low-temperature alcohol method was supposed to yield native serum proteins. Unfortunately, even though the albumins were still soluble at the isoelectric point they were denatured to the extent that they were poorly metabolized by the organism when injected. The low-temperature alcohol method has therefore been supplanted by a procedure involving the use of specific adsorbants and fractional precipitation with zinc salts at pH 7.4. The Zn^{++} ions are removed by means of a cation exchange resin. The following components have been identified in the fractions obtained in this method:

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[5] Cohn, Luetscher, Oncley, Armstrong, and Davis: *J. Am. Chem. Soc.*, **62**, 3396 (1940).

On Cation-Exchange Resin	*On Stroma of Red Cells*	*Precipitate with Zn^{++} at pH 7.4*	*Filtrate from Zinc at pH 7.4*
Prothrombin Prothrombin accelerator Leukocyte factor Lipoproteins	Isoagglutinins	Fibrinogen Immune globulins Other globulins β-Lipoproteins Cu-protein, etc.	Albumins Metal-combining globulin Glycoproteins Amylase Iodoprotein Esterase etc.

The serum albumin fraction so prepared is relatively heat-stable, does not contain any known virus contaminants, and is approximately as active osmotically as untreated human plasma.

USE OF THE ULTRACENTRIFUGE. Separation of the plasma proteins in the ultracentrifuge of Svedberg (see Chapter 1) has had somewhat limited applicability because of the superiority of other available methods, but has yielded valuable theoretical information. It has been found that bovine serum contains an albumin with a molecular weight of about 64,500 as estimated from the sedimentation data, together with several globulins. The two most abundant globulins have molecular weights of about 165,000.

ELECTROPHORESIS. The fractionation of the plasma proteins by the use of electrophoresis (see Chapter 1) was initiated largely by the pioneer work of Tiselius. In the electrophoretic separation of the plasma proteins, advantage is taken of the differences in mobility of the various protein ions present under the influence of a potential gradient. The protein solution is first dialyzed against a suitable buffer solution through cellophane tubing (Visking sausage casing) in order to equilibrate the two solutions with regard to their pH and conductivity. In an electrophoresis cell of the type shown in Fig. 105, one of the rectangular limbs and the bottom section are filled with the protein solution, while the other limb and the rest of the electrophoresis cell including the electrode vessels (not shown in the figure) are filled with the buffer solution serving as the outside fluid during dialysis. Sharp starting boundaries are produced by manipulating the cell sections. Reversible electrodes of the silver-silver chloride type, immersed in strong KCl solution, are connected with a suitable source of

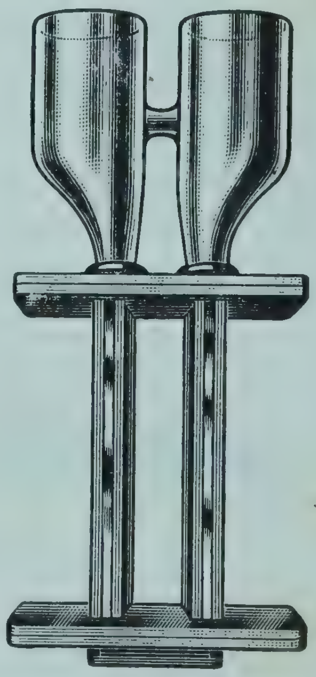
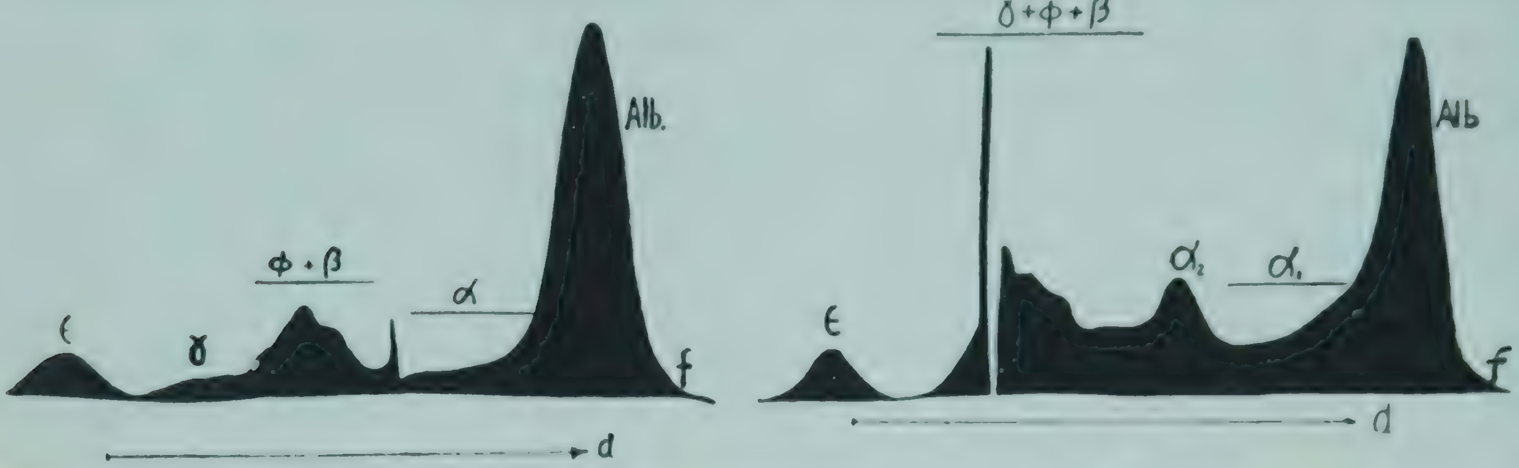
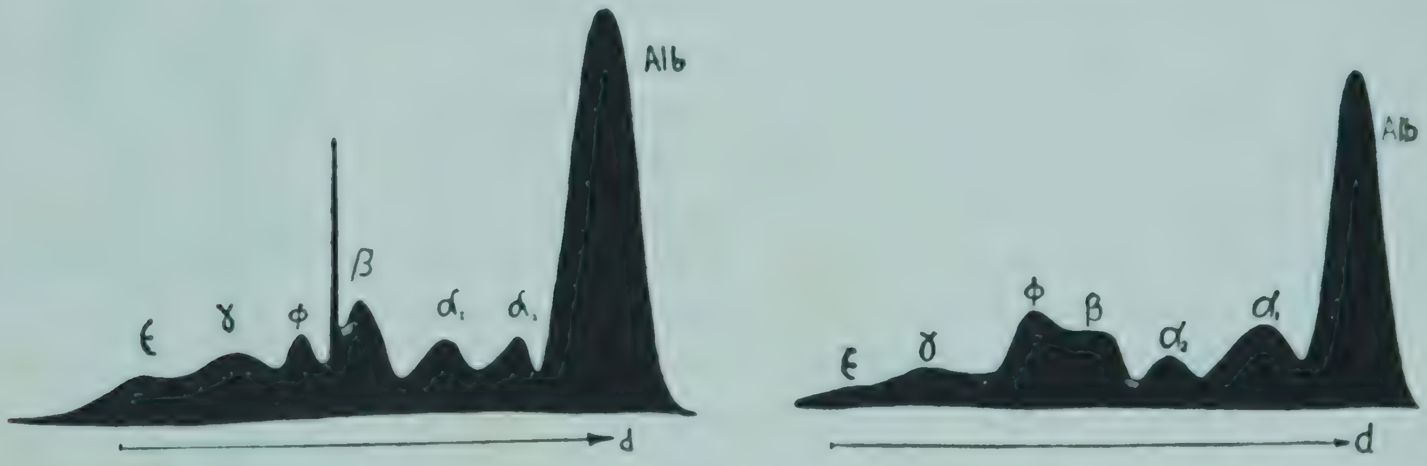


FIG. 105. TYPE OF CELL USED FOR ELECTROPHORESIS STUDIES ON PLASMA PROTEINS.
Courtesy, Klett Manufacturing Co., New York.

high-voltage direct current. Under the influence of the potential gradient thus produced (4 to 8 volts per cm.) the various species of protein ions present migrate downward in one limb of the cell and upward in the other, in the direction toward the anode at alkaline pH values, at a rate which is a function of the surface-charge density, i.e., the number of free positive (or negative) charges per area unit of the surface of the protein molecules. By so moving, the individual protein ion species form more or less well-defined boundaries arranged in the order of their respective electrophoretic mobilities. However, it is to be noted that there is a considerable amount of overlapping of the various protein regions so that only the *fastest* and the *slowest* components in a multicomponent system may, as a rule, be obtained in pure form by means of the moving-boundary technique. As the concentration of the individual proteins changes, the index of refraction of the medium likewise changes, and by suitable optical methods based upon this principle the migration of the various protein fractions may be followed (Longsworth, Svensson).



Rat *Chicken*



Human *Dog*

FIG. 106. ELECTROPHORETIC PATTERNS OF BLOOD PLASMA FROM VARIOUS ANIMAL SPECIES.

Courtesy, Deutsch and Goodloe, *J. Biol. Chem.*, **161**, 1 (1945).

The type of pattern obtained during electrophoresis of a mixture of components is illustrated by Fig. 106, which represents the electrophoretic patterns of various animal plasmas. The distance along the x axis is a measure of the relative velocity of movement of the various ion species present, and the height of the peaks corresponds to the difference in refractive index between the moving boundary and the adjacent fluid, the area under each curve being proportional to the amount of material present moving with an average velocity represented by the position of the peak along the x axis. Thus by this method it is possible not only to distinguish between ion species of different mobilities but also to estimate the relative amounts of each type of ion present.

Tiselius demonstrated that normal plasma contained at least five electrophoretically distinguishable components, which were identifiable as albumin, fibrinogen, and three globulins, designated α-, β-, and γ-globulin respectively, the α-globulin having the fastest velocity in the globulin group and the γ-globulin the slowest. From electrophoretic data, it is clear that while albumin and fibrinogen are reasonably homogeneous—the high sharp peak corresponds to a migration of protein ions the majority of which have the same net charge and weight—the globulin fraction consists of a number of types of ions which may be classed roughly into three groups but obviously include ion species of quite varying mobilities within any one group. Indeed, later workers have been able to show that the three globulin fractions of Tiselius may be resolved into further components which have been designated α_1- and α_2-globulin, β_1- and β_2-globulin, etc. In addition, stationary peaks, designated as δ and ϵ respectively, are observed in the ascending and descending boundary diagrams (see Fig. 106). These have been shown to represent boundary anomalies rather than additional protein components. The fibrinogen boundary, usually designated by the symbol ϕ, is situated between the β- and γ-globulin boundaries. Following the example of Longsworth, most workers employ a diethylbarbituric acid–sodium diethylbarbiturate buffer system of pH 8.6 and 0.1 ionic strength which yields the highest degree of resolution of human serum or plasma diagrams.

The type of quantitative distribution of the plasma proteins that is obtained by electrophoresis is illustrated in the table below:

DISTRIBUTION OF PROTEINS IN CITRATED PLASMA AS DETERMINED BY ELECTROPHORETIC ANALYSIS*

	Grams per 100 ml. of Plasma	*Per Cent of Total Protein*
Total protein	6.03	100
Albumin	3.32	55
α-Globulin	0.84	14
β-Globulin	0.78	13
γ-Globulin	0.66	11
Fibrinogen	0.43	7

* Adapted from data of Cohn, Oncley, *et al.*: *J. Clin. Invest.*, **23**, 417 (1944).

It is to be noted that the three main globulin components are here present in approximately the same concentration, and furthermore that the ratio of albumin to globulin (the so-called "A/G ratio") is 1.45. This value is to be contrasted with the value of approximately 2.0 or higher which is accepted as normal for fractionation by sodium sulfate precipitation.

There is no simple relation between the fractions of the plasma proteins obtained by salting out and those obtained by electrophoresis studies. Thus Cohn, McMeekin, *et al.* (*loc. cit.*) have reported that at 34 per cent saturation with ammonium sulfate, the protein fraction precipitating consisted largely of γ-globulin, one-third of which was euglobulin—i.e., precipitated on dialysis. At 40 per cent saturation with ammonium sulfate the fraction contained α- and β-globulins as well as some γ-globulin. This fraction likewise contained about one-third euglobulin. Increasing the saturation to 50 per cent yielded a precipitate which was free from γ-globulin but which contained both α- and β-globulins, with only a few per cent of the fraction insoluble in water. Thus the two methods of fractionation—salting out and electrophoresis—yield overlapping results and are not directly comparable. But it is to be noted that by suitable salting-out methods fractions can be prepared which are homogeneous electrophoretically and which correspond to the fractions obtained electrophoretically. Thus Pillemer and Hutchinson[6] have reported that at 0° C. and pH 6.7 to 6.9, fractionation of human serum proteins with 42.5 per cent methanol yields A/G ratios which compare favorably with those obtained by electrophoretic analysis.

Recently, methods for the separation of small amounts of proteins, e.g., serum or plasma, by electrophoresis in filter-paper strips (zone electrophoresis) have been developed by Durrum, Grassmann, Tiselius, and others. This technique, though not as strictly quantitative as the classical moving-boundary method of electrophoresis, promises to have widespread application in clinical routine work. Fig. 57 (p. 184) shows the photograph of a developed paper strip after serum electrophoresis and the corresponding curve obtained by photoelectric densitometry of the stained protein bands. It should be pointed out that in zone or paper electrophoresis the individual bands visible after treating the fixed strip with suitable stains—e.g., Amido Schwarz 10B—contain the actual protein components rather than represent their boundary against zones of different refractive index as in the moving-boundary method of electrophoresis (compare with diagrams in Fig. 106).

At the present time it would appear that the electrophoretic method of analysis of the plasma proteins has its greatest value in studying the relative amounts of the various components present and the changes which these components undergo during disease, with a view to possible aid in diagnosis; but fractional precipitation is of value in the large-scale preparation of the individual components for industrial or clinical use, the purity of the fractions being controlled by electrophoretic analysis.

Origin of the Plasma Proteins. The liver is usually considered to be the site of formation of the plasma proteins, although other parts of the body may also have a function in this connection. Fibrinogen synthesis appears to be dependent entirely upon the liver. When the liver is damaged experimentally by poisons, the fibrinogen content of the blood falls, returning to normal with liver regeneration and repair. If the blood of a normal animal is removed as much as possible and replaced by defibrinated blood, the fibrinogen level is restored to normal in a few hours; in the hepatectomized animal this restoration does not occur.

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[6] Pillemer and Hutchinson: *J. Biol. Chem.*, 158, 299 (1945).

Evidence relating the liver to the production of the other proteins of plasma is less definite. Liver atrophy is frequently found in experimental and clinical hypoproteinemia. Since the albumin fraction appears to suffer most under these conditions, it would appear that the liver is associated with albumin synthesis, and furthermore that the factors controlling the synthesis of albumin are different from those for globulin; indeed, there is considerable evidence that parts of the body other than the liver are concerned with plasma globulin formation.

The study of plasma protein production in experimental animals is facilitated by the use of the experimental technique known as plasmapheresis, in which whole blood is removed from an animal, the blood cells separated from the plasma and resuspended in a suitable isotonic protein-free medium, and returned to the animal's circulation. Thus a plasma-protein deficit is produced which may be maintained at any desired point, and the extent to which the animal attempts to restore the plasma-protein level to normal may be used as an index of plasma-protein formation under various experimental conditions. Using this technique, for example, it has been found that plasma protein production in the dog may be maintained at normal levels for many weeks by the intravenous administration of a mixture of purified amino acids as the sole source of dietary nitrogen, and that those amino acids in particular which have been found by Rose to be essential in the diet of the young growing rat (see Chapter 33) are likewise significant in the production of plasma protein in the dog.

There is increasing evidence that the plasma proteins are in continuous metabolic equilibrium with other proteins and amino acids of the body. This view, first postulated by Whipple and collaborators as the result of studies on protein regeneration in animals by the plasmapheresis technique, is supported by the work of Schoenheimer *et al.*,[7] using the nitrogen isotope N^{15}. When amino acids containing this isotope were fed to rats in nitrogen equilibrium, both the tissue proteins and the plasma proteins were found to incorporate the isotopic nitrogen rapidly and at about the same rate. On discontinuing isotope administration, the isotope gradually disappeared from the plasma proteins, the estimated half life of the plasma-protein molecule being about two weeks. These and other experiments indicate that the plasma proteins in an animal are not static but are subject to continuous influence by dietary and metabolic factors.

Functions of the Plasma Proteins. In addition to the specific physiological function of certain of the plasma proteins, such as the role of fibrinogen and prothrombin in blood-clotting and the role of the plasma globulins in immunological reactions, certain general functions of the plasma proteins are recognized. These may be classified as nutritive and physicochemical.

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[7] Schoenheimer, Ratner, Rittenberg, and Heidelberger: *J. Biol. Chem.*, **144**, 541, 545 (1942).

NUTRITIVE FUNCTION. The nutritive function of the plasma proteins has been definitely established largely through the work of Whipple and his associates. They have shown that the nitrogen (and protein) requirements of the fasting animal can be adequately supplied by the intravenous injection of plasma protein. Since this injected protein disappears from the circulation in a short time, it is presumably metabolized. Analyses of urinary nitrogen excretion after plasma-protein injection indicate an increased catabolism of protein. Whether this means that the plasma proteins are directly utilized metabolically or are first converted into tissue protein is not known; such a distinction would appear to be relatively unimportant in view of the existence of such a dynamic equilibrium between tissue proteins and plasma proteins as has already been postulated.

PHYSICOCHEMICAL FUNCTIONS. The physicochemical functions of the plasma proteins are equally important. Chief of these appear to be to aid in the maintenance of a normal blood volume and in the maintenance of a normal water content in the tissue fluids. By virtue of their colloidal dimensions the plasma proteins cannot normally diffuse through the blood capillary membranes into the relatively protein-free tissue fluids. They thus exert an osmotic pressure which acts as a force tending to hold a certain volume of water within the blood. This colloidal osmotic pressure—or *oncotic pressure*, as it is sometimes called—has a magnitude of about 25 mm. Hg. Although this is much less than 1 per cent of the total osmotic pressure of the plasma, nevertheless it becomes the dominant osmotic force in the blood capillaries, since the other plasma constituents are freely diffusible across the capillary membranes.

Of the various plasma proteins, albumin is by far the most significant in connection with osmotic pressure, being estimated to account for about 80 per cent of the total osmotic pressure of the plasma proteins. Gram for gram, albumin is at least twice as effective osmotically as globulin, largely because of its relatively lower molecular weight. It has been shown that 1 g. of plasma albumin is responsible for the retention of roughly 20 ml. of water in the blood. A loss of plasma protein, and particularly of plasma albumin, therefore leads to a diminished blood volume, and this is presumably a major cause of the symptoms of hemorrhagic shock. The administration of plasma protein, especially plasma albumin concentrates, has been shown to be of considerable value in both experimental and clinical shock.

The colloidal osmotic pressure of the plasma proteins is opposed by the filtering force of the blood pressure, which tends to drive water and dissolved substances across the capillary membranes into the tissue fluids. At the arterial end of a blood capillary, the blood pressure exceeds the colloidal osmotic pressure, and fluid is forced from the blood into the tissues; at the venous end the reverse action takes place, fluid entering the blood from the tissues. Normally there is a balance between these two opposing forces, and fluid distribution between the blood and tissues is normal. If this balance is upset by such conditions as a low plasma-protein content or an increased permeability of the capillary walls to protein, excessive amounts of water will accumulate in the tissues (edema). A plasma-protein deficit is therefore an important (although by no means the only) cause of edema. Edema due to plasma-protein deficit has been successfully treated by measures which restore the plasma-protein level to normal.

A second physicochemical function of the plasma proteins is in connection with acid-base balance. Because the plasma proteins in solution at pH 7.4 are on the alkaline side of their isoelectric points, they exist to a certain extent as alkali salts. They thus act similarly to the alkali bicarbonate of the blood in furnishing base for the neutralization of acid, and indeed it has been shown that in the plasma the proteins are second to bicarbonate in importance in this respect.

Other functions of the plasma proteins are also recognized. They aid in promoting the mobility of the corpuscles, since red cells settle more rapidly in plasma than in protein-free isotonic solutions. The globulin fraction appears to be the carrier of the immune substances of the blood, and indeed the isolation and concentration of immune substances from human plasma is an important phase of fractionating the plasma proteins. The plasma proteins also combine with certain drugs and have thus been ascribed a vehicular function, although the precise significance of this property is not yet known.

The Erythrocytes. The erythrocytes or red cells make up about 45 per cent by volume of the blood. Variations from this value are frequently encountered; they are associated with changes in either the number of cells per unit volume of blood, or the size of the individual cells, or both. The relative cell volume of blood is determined with the hematocrit, a graduated tube which may be filled with whole blood and centrifuged. The volume of packed cells relative to the initial volume of blood is a measure of the cell volume. The top layer of packed cells is frequently almost colorless because of the predominance of leukocytes, which are not so heavy as the erythrocytes. The same phenomenon is noted when blood is allowed to clot without agitation; the lighter superficial portion of the clot is then known as the "buffy coat." The rate at which the erythrocytes settle, the so-called *sedimentation rate*, is applied clinically. Why the red cells settle out at different rates in the blood of various pathological conditions is not known. However, the rate is more rapid in tuberculosis, cancer, and acute inflammations. Newborn infants have a very slow rate, whereas normal men have a slower rate than normal women. The rapidity of the rate increases in menstruation and in normal pregnancy.

The erythrocytes are responsible for the opacity of blood. If blood is diluted with water this opacity disappears in a few moments, the fluid becomes translucent, and the blood is said to be hemolyzed or laked. On diluting blood with 0.9 per cent sodium chloride solution, however, no hemolysis occurs. The explanation for this lies in the osmotic behavior of the erythrocytes. Each cell may be considered as a miniature osmometer, the water content of the cell depending upon the osmotic pressure both of the cell contents and of the surrounding medium. If, for example, the cell is in a medium such as 0.9 per cent sodium chloride solution, which has the same osmotic pressure as the cell contents, the water content of the cell will not change, nor will its size, and the medium is said to be *isotonic* with the cell. If the osmotic pressure of the medium is greater than that of the cell contents, water will be abstracted from the cell, it will decrease in size, and the solution is said to be *hypertonic*. A solution which has a lower osmotic pressure than the cell contents is *hypotonic;* in such a solution, the cell will absorb water and swell, the extent of swelling depending upon the degree of hypotonicity. In a sufficiently hypotonic solution the swollen erythrocyte loses its ability to retain hemoglobin, and hemolysis results. The osmotic pressure at which hemolysis occurs is known clinically as the fragility point, and its determination has a certain diagnostic value.

Other agencies besides osmotic-pressure differences will bring about hemolysis of erythrocytes. These include alkali, ether, chloroform, soaps, bile salts, saponins, certain bacterial toxins, and snake venoms. In these instances hemolysis must be attributed to the actual modification or destruction of the cell stroma.

Human erythrocytes are nonnucleated biconcave disks with an average diameter of about 8 μ (1 μ = 0.001 mm.). Mammalian erythrocytes vary in size from species to species, ranging from 2 μ to about 9 μ in diameter. In the blood of birds, fishes, amphibians, and reptiles the erythrocytes are ordinarily more or less elliptical, biconvex, and nucleated.

The number of erythrocytes present in human blood depends upon many factors, such as age, sex, altitude, exercise, etc. It is usually considered that the blood of a normal adult male contains 5,000,000 erythrocytes per cmm.; for a normal adult female the count is 4,500,000. Increased red-cell count is noted after blood transfusion, during residence at high altitudes, and after strenuous physical exercise; in the latter case a count of 7,040,000 has been observed. An increase is also noted in starvation, after partaking of food, after cold or hot baths, after massage, after partial asphyxia, and after fright, as well as after the administration of certain drugs and accompanying certain diseases, such as cholera, diarrhea, dysentery, and yellow atrophy of the liver. In polycythemia counts as high as 11,000,000 have been noted, and values almost as high have been observed in cyanosis. Experimentally, polycythemia may be produced in animals by the inclusion of cobalt salts in the diet. A decrease in the number of erythrocytes occurs in the different forms of anemia, values as low as 500,000 per cmm. or lower having been noted in pernicious anemia.

Erythrocytes possess the property of grouping together in masses, or "clumping." This action occurs normally on a microscopical scale, and since the cell aggregates settle faster than the discrete cells, clumping is a major factor in determining the sedimentation rate of the blood cells, a characteristic of blood the measurement of which was shown by Fåhraeus to have considerable clinical value. If the clumping power of the cells is so enhanced as to produce macroscopically visible clumps, the process is called agglutination. Cells other than erythrocytes (e.g., bacteria) possess this property; when spoken of in connection with the blood the term *hemagglutination* is frequently used. Observation of hemagglutination is the basis for the establishment of blood types, so essential in connection with blood transfusion; indeed, it is agglutination of erythrocytes which renders incompatible the bloods of donor and recipient. A substance which will bring about hemagglutination is said to contain hemagglutinins; if these are species specific they are known as isohemagglutinins. In human plasma the isohemagglutinins are associated largely with the β- and γ-globulin fractions. Hemagglutinins are abundant in the vegetable kingdom; for a demonstration of hemagglutination, see p. 486.

Composition of Red Cells. The red cells contain approximately 65 per cent water and 35 per cent solids. Of the solids, the red chromoprotein hemoglobin comprises about 32 of the 35 per cent; most of the remaining 3 per cent forms the stroma of the red cell, which consists largely of protein, phospholipide, and cholesterol. Inorganic ions in the red cell include potassium, chloride, bicarbonate, and phosphate; of these, potassium is present in largest amount, being comparable quantitatively, and in certain respects equivalent physiologically, to the sodium of the plasma. Organic constituents include various phosphate esters and certain enzymes, such as phosphatases and carbonic anhydrase; this latter is of particular significance in connection with the function of the red cells in the carriage of carbon dioxide by the blood (see Chapter 24). Certain diffusible substances such as glucose and urea are found equally distributed between cells and plasma when concentrations are expressed in terms of the water present; it is important to remember in such comparisons as this that on a percentage basis the amount of water in the cells is much less than that in the plasma.

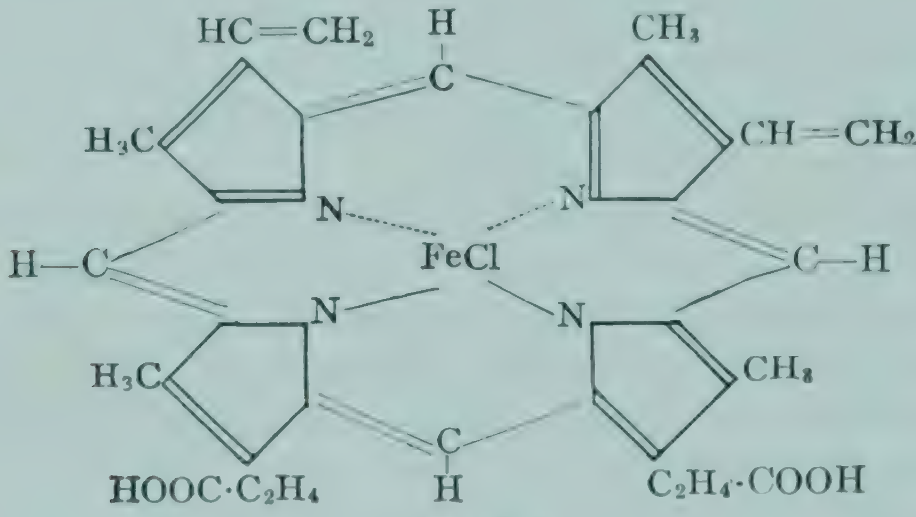
Although erythrocytes contain oxidative enzymes (the "yellow enzyme" of Warburg was first isolated from horse red cells), the respiratory metabolism of these cells is very small. The major functions of the red cell appear explicable on a purely physicochemical basis in terms of the role of hemoglobin and other substances present in the transport of oxygen and carbon dioxide by the blood.

Hemoglobin. Hemoglobin, the red coloring matter of the blood, is the most abundant protein in blood, being usually found to the extent of about 14 to 16 g. per 100 ml. of whole blood. It is normally found entirely within the erythrocytes, from which it may be released by suitable hemolytic agents. It is a readily crystallizable conjugated protein, consisting of a colorless protein portion known as globin, which makes up about 96 per cent of the hemoglobin molecule, and a colored nonprotein portion or prosthetic group which has been shown to be an iron-containing compound belonging to the class of porphyrins. The chemical nature of globin has been discussed in Chapter 6.

The molecular weight of hemoglobin is approximately 67,000, concordant results being obtained by a variety of methods. The iron content of crystalline human hemoglobin is 0.340 per cent. Since this corresponds to a minimal molecular weight of 16,700 on the basis of one Fe atom per molecule, it has been concluded that the hemoglobin molecule contains four Fe atoms, and the physicochemical behavior of hemoglobin is in agreement with this conclusion.

It is now clearly established that several types of human hemoglobin exist. In the prenatal period, the blood contains hemoglobin distinctly different from the adult type. It is called fetal hemoglobin and is recognized by its alkali resistance. Fetal hemoglobin gradually disappears during the first year of life except in certain chronic anemia states in which it continues to be present in the blood. *Sickle-cell anemia*, a disease confined almost exclusively to the Negro, is characterized by an abnormal hemoglobin and a peculiar behavior of the erythrocytes. The cells acquire a crescent shape when subjected to low oxygen tension. Sickle-cell hemoglobin and normal hemoglobin have the same oxygen dissociation curve, similar crystal forms, and identical solubilities, but differ in their electrophoretic mobilities. This difference is sufficient to produce a serious disease which is transmitted by inheritance.

Hemoglobin is readily separated into its protein and prosthetic-group components by treatment with acid, the globin usually being denatured in the process, and the iron-containing portion being obtainable under the proper conditions in the form of an insoluble crystalline compound known as hemin. The formation of hemin crystals is frequently used as a test for blood because of their characteristic appearance and the ease with which they may be obtained. Hemin has been synthesized and its structure is represented by the following formula:



Hemin

It can be seen that the hemin molecule contains iron in the trivalent form, and that the rest of the molecule consists essentially of four substituted methyl-pyrrole rings linked together by —CH= bridges. Such a molecule is known as a metalloporphyrin, the metal in this case being iron. Various isomers of the porphyrin molecule are known, differing in the relative position of the substituting side chains, the isomer found in hemin being called protoporphyrin. To indicate the valence of the iron, which is of importance, it has been suggested that the terms ferroprotoporphyrin and ferriprotoporphyrin be used. Thus hemin is the chloride of ferriprotoporphyrin. The term *heme* is also frequently used as equivalent to ferriprotoporphyrin, hemin then being the chloride of heme. The older term "hematin" has been largely abandoned because of its indefinite connotation; it is roughly equivalent to heme.

When hemin is treated with a suitable reducing agent the ferric iron is reduced to the ferrous state. The resulting ferroprotoporphyrin or reduced heme readily combines with undenatured globin to form a compound which is closely similar to, if not identical with, natural hemoglobin. Thus hemoglobin is a combination of globin with reduced heme, the iron in the hemoglobin molecule being in the ferrous state. The nature of the combination between the protein and the prosthetic group is not known.

Heme and reduced heme have the ability to combine with other nitrogen-containing compounds besides globin and denatured globin; such substances include other proteins, ammonia, cyanide, nicotine, pyridine, etc. These combinations are known in general as hemochromogens. Spectroscopic and other studies of the various natural and synthetic hemochromogens have thrown much light on our knowledge of the chemical behavior of heme and hemoglobin, and hemochromogen formation has been utilized for the qualitative detection and quantitative determination of hemoglobin. The occurrence of heme in nature as the prosthetic group of the enzymes catalase and peroxidase, and in the cytochromes, has already been discussed (see Chapter 12).

Combination of Hemoglobin with Oxygen. A major physiological function of hemoglobin is based upon its ability to react reversibly with oxygen. This reaction may be written as follows:[8]

$$\underset{\textbf{Hemoglobin}}{Hb} + O_2 \rightleftarrows \underset{\textbf{Oxyhemoglobin}}{HbO_2}$$

Under optimal conditions, 1 g. of hemoglobin will combine with 1.36 ml. of oxygen.[9] The product of this reaction, oxyhemoglobin, is as well characterized a compound as hemoglobin, and is quite stable with respect to its oxygen content as long as there is sufficient oxygen present to prevent the reaction from going to the left. Oxyhemoglobin may be crystallized, and a specific form of crystal is obtained from the blood of each individual animal species (see Figs. 107 and 108). Reichert and Brown studied oxyhemoglobin crystals prepared from the blood of more than 100 species of animals from the point of view of their crystallographic characteristics. Species differences are not confined to crystal form only; Barcroft and others have shown that oxyhemoglobin from various sources may differ in spectroscopic characteristics and in affinity for oxygen. Since hemin crystals are identical no matter from what species the blood is obtained, the species differences in oxyhemoglobins must presumably be related to differences in the globin portion of the molecule.

The reversibility of the reaction between hemoglobin and oxygen to form oxyhemoglobin implies that the relative amounts of hemoglobin and oxyhemoglobin present in blood will depend upon the concentration of oxygen present, which in turn is proportional to the oxygen tension (Henry's Law). The oxygen tension within the red cells is determined largely by the oxygen tension of the plasma, since oxygen is freely diffusible across the red-cell membrane.

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[8] The reaction is written in the form given for purposes of simplicity. It has already been pointed out that there are four Fe atoms per molecule of hemoglobin, and it is known that hemoglobin combines with oxygen in the proportion of one O_2 molecule per atom of Fe. Thus the reaction should really be written: $Hb_4 + 4O_2 \rightleftarrows Hb_4O_8$. Furthermore, this latter reaction provides for the possibility of intermediate compounds such as Hb_4O_2, Hb_4O_4, and Hb_4O_6, the formation of which would depend on the amount of oxygen available; and Pauling and others have shown that the sigmoid curve relating oxyhemoglobin formation to oxygen tension is explicable on the basis of equilibria involving the formation of such intermediate stages in oxygenation.

[9] Bernhart and Skeggs: *J. Biol. Chem.*, **147**, 19 (1943).

The relation between the degree of oxygenation of hemoglobin and the oxygen tension is usually expressed graphically in the form of a

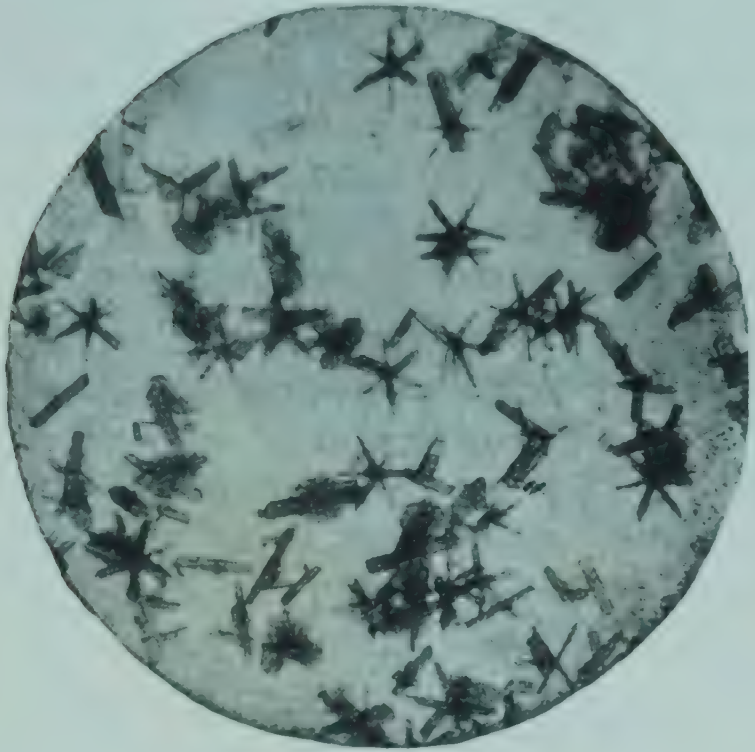

FIG. 107. OXYHEMOGLOBIN CRYSTALS FROM BLOOD OF THE RAT.

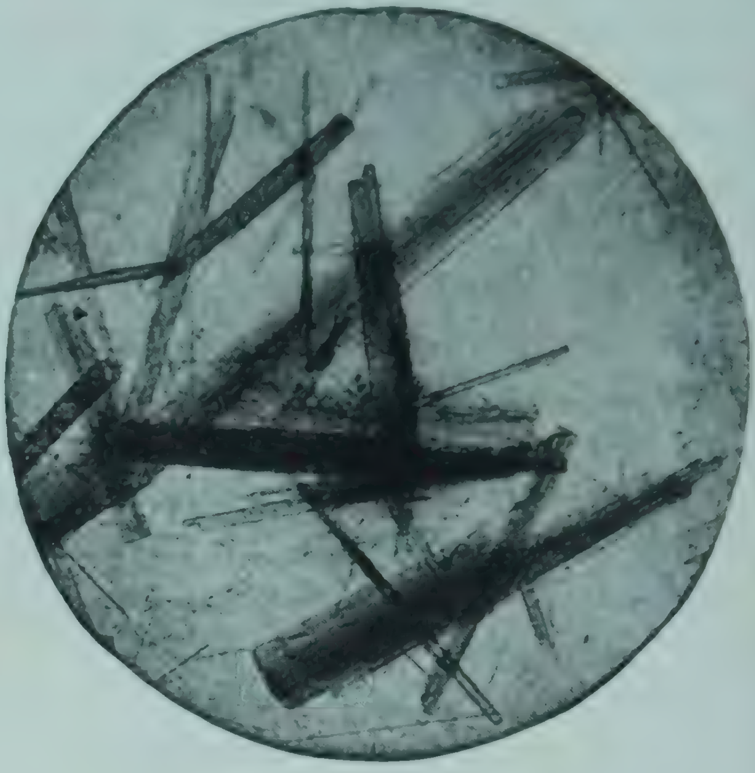

FIG. 108. OXYHEMOGLOBIN CRYSTALS, FROM BLOOD OF THE DOG.

These illustrations were reproduced from photomicrographs furnished by the late Prof. E. T. Reichert of the University of Pennsylvania.

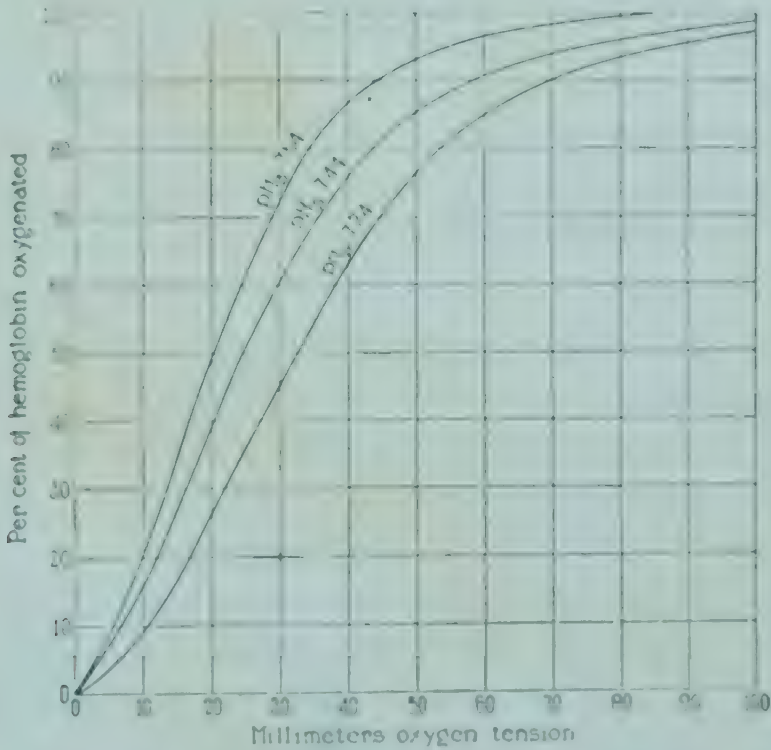

FIG. 109. OXYGEN DISSOCIATION CURVE FOR HEMOGLOBIN IN BLOOD AT VARIOUS VALUES OF SERUM pH(pH_s).

Courtesy, Van Slyke and Peters: *Quantitative Clinical Chemistry.*

curve, the *oxygen dissociation curve*, which is illustrated by Fig. 109. The effect of such variables as pH and temperature on the ability of hemoglobin to combine with oxygen may be studied by noting their influence on the shape of the oxygen dissociation curve. From the curve it can be seen that when blood is in equilibrium with ordinary room air (oxygen tension = *ca.* 150 mm.Hg) or with the alveolar air of the lungs (oxygen tension = *ca.* 90 to 100 mm.Hg), practically all of the hemoglobin is oxygenated, i.e., the per cent saturation of the blood with oxygen is from 95 to 100 per cent. As the blood courses through the tissue capillaries, however, where there is a constant demand for oxygen, oxygen diffuses out of the plasma into the tissue fluids and the plasma oxygen tension falls to about 40 mm.Hg. At this tension the oxyhemoglobin has yielded up about one-quarter of its combined oxygen by dissociation; thus normal venous blood contains about 75 per cent oxyhemoglobin and 25 per cent hemoglobin, or is 75 per cent saturated with oxygen. Further lowering of the oxygen tension leads to increased dissociation of oxyhemoglobin; in the extreme condition of cyanosis only a minor portion of the pigment may be oxygenated. The sigmoid shape of the curve is considered to have some physiological significance. As the oxygen tension is lowered (i.e., as the demand for oxygen becomes greater), a given de-

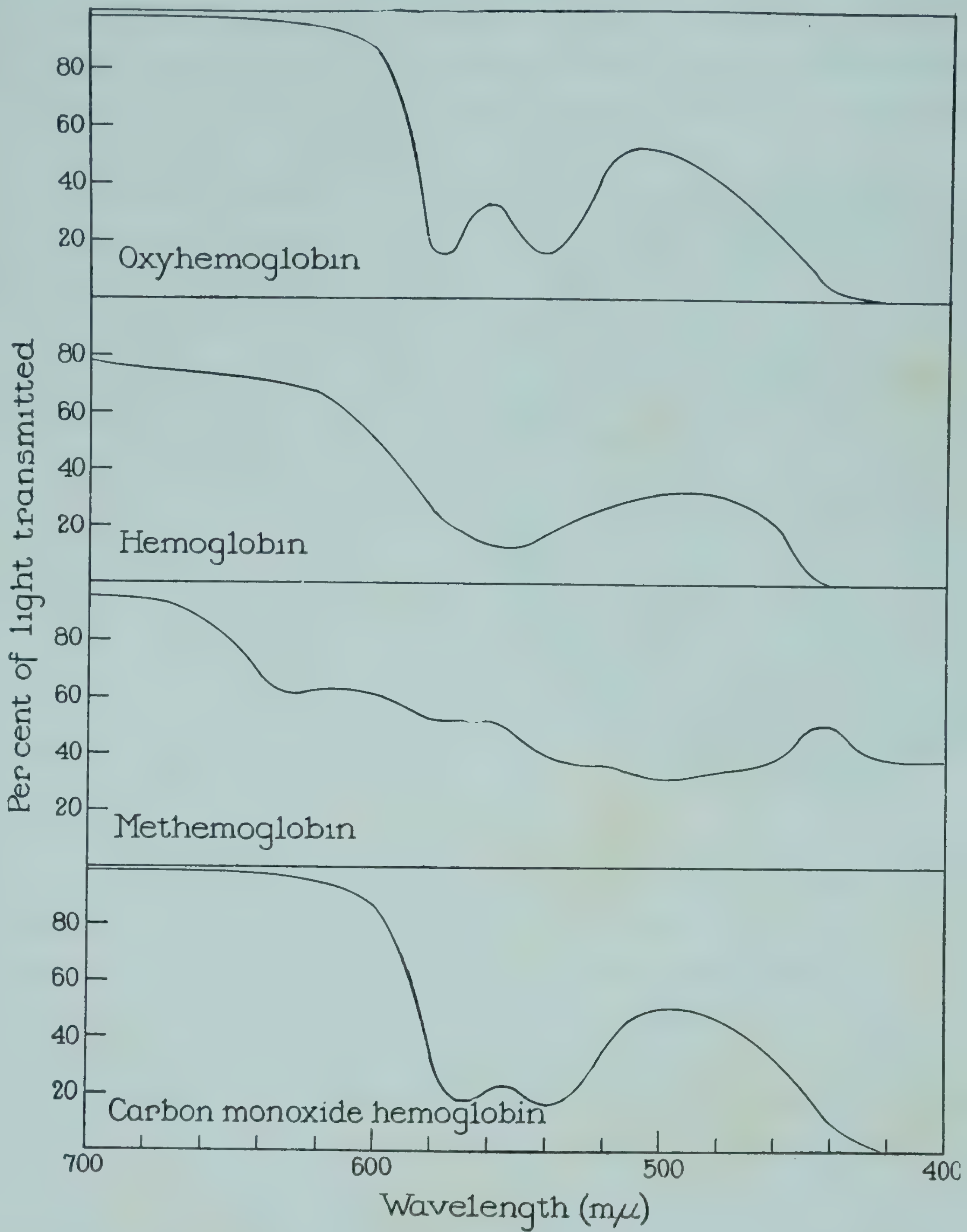

FIG. 110. RELATION BETWEEN WAVELENGTH AND LIGHT ABSORPTION FOR HEMOGLOBIN AND OTHER BLOOD PIGMENTS, ALL AT A CONCENTRATION OF 1 G. PER LITER AND AT A SOLUTION DEPTH OF 1 CM.

crease in tension produces a greater dissociation of oxyhemoglobin than is produced for the same decrease in tension at high tensions.

The physiological function of hemoglobin is not confined to oxygen transport; it is of equal significance in the carriage of carbon dioxide by the blood. This function is discussed in detail in Chapter 24.

Other Reactions of Hemoglobin. In addition to its ability to react with oxygen, hemoglobin will react with a variety of other compounds, such as carbon monoxide, nitric oxide, hydrogen sulfide, ferricyanide, etc., the product in most cases being a colored compound which, like hemoglobin and oxyhemoglobin, may usually be identified by its characteristic absorption spectrum. The absorption spectra of hemoglobin and some hemoglobin derivatives are shown in Fig. 110 as they are obtained quantitatively with the spectrophotometer. The actual extent to which the light is absorbed is indicated by the data in Fig. 110.

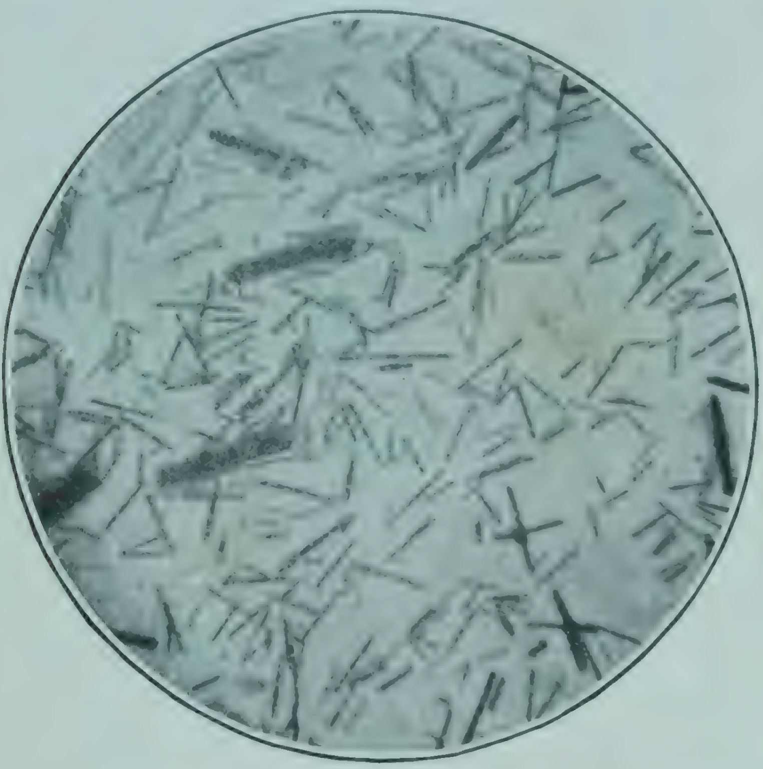

FIG. 111. CARBON MONOXIDE HEMOGLOBIN CRYSTALS FROM OX BLOOD.
Courtesy, Boor: *J. Gen. Physiol.*, **13**, 307 (1930).

The combination between hemoglobin and carbon monoxide to form carbon monoxide hemoglobin is of interest because of the constant presence of small amounts of carbon monoxide in city air. Carbon monoxide hemoglobin is a bright red pigment which may be crystallized (see Fig. 111). Carbon monoxide has about 200 times the affinity for hemoglobin that oxygen has; furthermore, the formation of carbon monoxide hemoglobin prevents hemoglobin from combining with oxygen, presumably because both gases compete for the same spot in the hemoglobin molecule. Carbon monoxide will in fact displace oxygen from oxyhemoglobin, as this reaction indicates:

$$HbO_2 + CO \rightleftarrows HbCO + O_2$$

This reaction ordinarily proceeds from left to right; it is however reversible if the concentration of oxygen is made high enough, and in this fact lies the therapy for carbon monoxide poisoning. Carbon monoxide hemoglobin is not toxic in itself; death from carbon monoxide poisoning is presumably due to failure of sufficient oxygen to reach the tissues because of the decreased oxygen-carrying capacity of the blood when a significant proportion (40 to 60 per cent or more) of the total hemoglobin is in the form of carbon monoxide hemoglobin. The anoxia of carbon monoxide poisoning is not due entirely to passive blocking of oxygen transport by hemoglobin; the oxyhemoglobin present in blood containing much carbon monoxide hemoglobin has been shown to be less efficient in releasing oxygen to the tissues under a given gradient of oxygen tension. The blood of city dwellers regularly contains over 1 per cent of the total hemoglobin as carbon monoxide hemoglobin; tobacco-smoking may increase this to 5 per cent.

When hemoglobin is treated with certain oxidizing agents, either *in vitro* or *in vivo,* the substance methemoglobin is formed. This is a brown pigment which differs from hemoglobin and oxyhemoglobin in that the iron is in the *ferric* form, i.e., methemoglobin is a combination of heme, or ferriprotoporphyrin, and globin. Methemoglobin is usually detected by means of its characteristic absorption spectrum (see Fig. 110). It does not combine with either oxygen or carbon monoxide, but does form a colored cyanide derivative. There is little or no methemoglobin in normal blood; the appearance of a methemoglobinemia is noticed in certain diseases and after the administration of certain drugs, among them sulfanilamide. Methemoglobin is not toxic; its presence in blood simply means a proportional reduction in the oxygen-carrying capacity of the blood. The clinical induction of a moderately severe methemoglobinemia has been proposed as an aid in the treatment of cyanide poisoning, since methemoglobin combines with cyanide and may thus prevent the latter from reacting with enzymes in the tissues.

Origin of Hemoglobin. Hemoglobin is synthesized in the body from the ingredients of the diet; iron, a source of the porphyrin nucleus, and amino acid precursors of the protein globin are of obvious importance in this connection. It is known that inorganic iron of the diet cannot be converted into hemoglobin without the presence of catalytic traces of copper; the way in which copper functions is not known.[10] Studies on the origin of the porphyrin nucleus and on the significance of dietary amino acids in hemoglobin synthesis have yielded little definite information; work with isotopes indicates that glycine and acetic acid may be precursors of the porphyrin ring (see also Chapter 33). In the adult animal the erythrocytes are formed in the bone marrow; in the embryo the liver is an important source of red-cell production, and the possibility that in the adult the liver retains a vital part in hemoglobin synthesis and erythrocyte formation cannot be overlooked. In the nutritional anemias there is usually a dietary deficiency leading to impaired hemoglobin formation; when the deficiency is corrected the anemia disappears. In pernicious anemia there appears to be lacking a factor which is necessary for the production of mature erythrocytes. This factor, vitamin B_{12} or cyanocobalamin (see p. 1207), has been isolated and found to contain 4.5 per cent of cobalt. Interestingly, both vitamin B_{12} and folic acid correct the maturation defect in pernicious anemia, but folic acid fails to prevent the neurological complications which commonly occur. It is believed that the absorption of vitamin B_{12} is defective in pernicious anemia and that this is due to the lack of a factor secreted by the stomach which is known as the *intrinsic factor of Castle.* This would explain why vitamin B_{12} is exceedingly effective when given parenterally even in minute doses, but relatively ineffective when administered orally.

The White Cells. The white corpuscles (or leukocytes) of human blood differ structurally from the red corpuscles (or erythrocytes) in many particulars, such as being larger in size, containing at least a single nucleus, and possessing ameboid movement. They are typical animal cells and therefore contain the following substances which are customarily present in such cells: Proteins, fats, glycogen, purines, enzymes, phosphatides, cholesterol, inorganic salts, and water. Compound proteins make up the chief part of the protein quota of leukocytes, the nucleoproteins predominating. Powerful proteolytic and glycolytic enzymes are also present. It is believed that there are two proteolytic enzymes in leukocytes, one active in alkaline solution and present in the polynuclear cells, and the other active in acid medium and present in mononuclear cells. It is claimed that the granular leukocytes originate in the bone marrow, whereas the nongranular leukocytes (lymphocytes) have a lymphatic origin (lymph glands or lymphoid tissue); this matter of origin is uncertain. The normal number of leukocytes in human blood varies between 5,000 and 10,000 per cmm. The ratio between the leukocytes and erythrocytes is about 1:350 to 500.

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[10] Glass: *Copper Metabolism,* Baltimore, Johns Hopkins University Press, 1950; Marston: *Physiol. Revs.,* **32,** 66 (1952).

A leukocytosis is said to exist when the number of leukocytes is increased for any reason. Leukocytoses may be divided into two general classes: the physiological and the pathological. Under the physiological form would be classed those leukocytoses accompanying pregnancy, parturition, digestion, and excessive physical exercise, as well as those due to mechanical and thermal influences. Leukocytosis is also associated with such emotional states as fear, rage, or apprehension. The leukocytoses spoken of as pathological are the inflammatory, infectious, posthemorrhagic, toxic, and experimental forms, as well as the type which accompanies malignant disease.

Chylomicrons. Blood contains myriads of spherical particles, about 0.5 to 1.0 micron in diameter, which are highly refractive and show Brownian movement. These were first recognized by Boyle in 1665, but were later described more fully by Müller who called them hemaconia or blood dust. However, Gage showed them to be minute fat globules, and curves of the chylomicron counts following the ingestion of fat have been studied in digestion experiments.

It has long been known that hyperlipemia is common in diabetes and that the incidence of atherosclerosis is high in this disease. The relationship of steroids in the blood to arterial degeneration has aroused intense interest.[11] Evidence is accumulating which suggests that steroid macromolecules may have an important causative role in atherosclerosis. The original finding of Hahn[12] (which has been repeatedly confirmed) that heparin has a striking effect on reducing postprandial hyperlipemia may perhaps be of both theoretical and clinical significance.

Blood Coagulation. When blood is withdrawn from a vein and put into a test tube, it will clot solidly in less than 10 minutes. After about 30 minutes the coagulum begins to retract, with the expression of a clear serum. These seemingly simple phenomena are the result of a complex series of reactions. The actual clot is due to the conversion of a soluble protein, fibrinogen, to fibrin, which separates as fibrils forming a meshwork that incloses both the cellular and plasma elements of the blood. Fibrinogen is changed to fibrin by the action of the enzyme thrombin. For the formation of this clotting factor, at least five agents have been shown to be essential: prothrombin, labile factor, calcium, thromboplastinogen, and platelets.

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[11] Gofman, Jones, Lindgren, Lyon, Elliott, and Strisower: *Circulation*, **2**, 161 (1950).

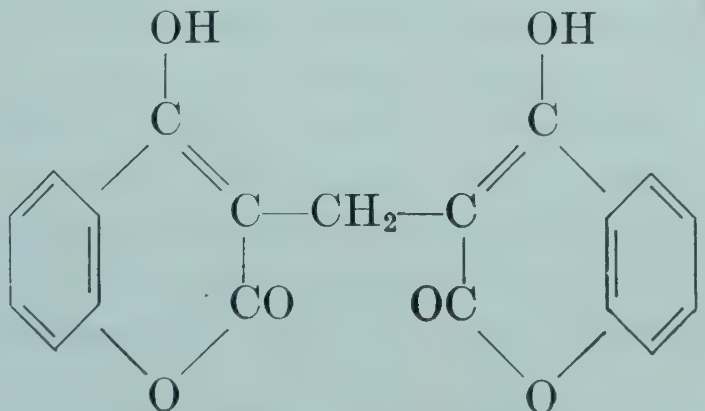
[12] Hahn: *Science*, **98**, 19 (1943).

PROTHROMBIN. Of the five basic thrombinogenic factors, prothrombin is the most clearly characterized and differentiated as a functional entity. It is a protein containing about 4 per cent carbohydrate. Its proenzyme nature is indicated by the fact that it becomes inactivated at 58 to 60° C. It is quantitatively removed from oxalated plasma by adsorbents such as tricalcium phosphate and barium sulfate and may be completely recovered from these adsorbents by elution with sodium citrate.

Vitamin K is essential for the synthesis of prothrombin, but the amount needed is exceedingly small. An adult dog requires only about 0.5 μg. of natural vitamin K per kg. of body weight daily to maintain a normal level of prothrombin. It is likely that the human requirements are about the same. The action of vitamin K can be satisfactorily explained by postulating that it functions as a prosthetic group combining with an *apoenzyme* (Æ) to form the *holoenzyme* (Æ·K), and that it is this enzyme which produces prothrombin. A deficiency of either apoenzyme or vitamin K results in a diminution of the holoenzyme and therefore in a decrease of the body's capacity to produce prothrombin. In the newborn a deficiency of vitamin K can easily occur due to inadequate intake. In the adult faulty absorption of vitamin K from the intestines is the most common cause for deficiency. Vitamin K_1, which is fat-soluble, requires bile salts for its absorption. Any condition therefore, such as biliary obstruction, in which bile does not enter the intestinal tract will bring about an inadequate absorption of vitamin K.

While the concept of an apoenzyme is still largely an hypothesis, the hypoprothrombinemia observed in certain liver diseases which is refractory to vitamin K is satisfactorily explained by the assumption that the apoenzyme is diminished. Likewise, congenital hypoprothrombinemia, which is unaffected by vitamin K, can be attributed to a lack of the factor which with vitamin K forms the enzyme that produces prothrombin.

Hypoprothrombinemia can be produced by a number of compounds which act as anti-vitamin-K agents. The best known of these is dicoumarol [3,3′-methylenebis(4-hydroxycoumarin)] which Link isolated from spoiled sweet-clover hay. It may be postulated that dicoumarol depresses the production of prothrombin by replacing vitamin K in the enzyme system



Dicoumarol

Æ K according to the equation: $Æ\,K + D \rightleftarrows Æ\,D + K$, where D stands for dicoumarol. The reaction is reversible and therefore the inhibitory action of dicoumarol can be counteracted by administering vitamin K. Dicoumarol and several other vitamin K antagonists are employed therapeutically to produce hypoprothrombinemia as a means of lessening the tendency to thrombosis.

Prothrombin is readily and accurately determined by means of the prothrombin time. In this procedure an excess of a standardized tissue extract such as acetone-dehydrated rabbit brain is added to recalcified plasma, and the clotting time is determined. By means of a curve, the prothrombin time can be expressed as per cent of normal clotting time. Under the conditions of the test, the clotting or prothrombin time is a direct measure of the prothrombin concentration. A second method known as the two-stage procedure consists in diluting plasma, after it has been defibrinated with a minute amount of thrombin, and converting all the prothrombin to thrombin by adding thromboplastin and calcium. The thrombin is determined by the speed with which it coagulates a fixed amount of fibrinogen.

Research indicates that only part of the prothrombin in human blood is in the active form; the remainder is present in a precursor state to which the name prothrombinogen has been given. In the blood of the newborn baby, prothrombinogen is lacking, but the concentration of active prothrombin is the same as in adult blood.[13] The prothrombin time, which measures only free or active prothrombin and is uninfluenced by prothrombinogen, is therefore normal. Since the two-stage method does not distinguish between free and inactive prothrombin but determines only total prothrombin, the concentration as determined by this method is only about one-third of the adult level. Since the newborn baby with a normal prothrombin time has no bleeding tendency, irrespective of the low prothrombin shown by the two-stage method, it may be concluded that only active prothrombin participates in hemostasis, and that therefore the prothrombin time is the more reliable test.

LABILE FACTOR. When human oxalated plasma is stored at 4° C., the prothrombin time becomes progressively prolonged. Since the addition of fresh plasma deprothrombinized by adsorption with $Ca_3(PO_4)_2$ restores the prothrombin time of stored plasma to normal, it must be concluded that the disappearance of a factor other than prothrombin must account for the loss of prothrombin activity during storage. This agent has been named *labile factor*, but is also known as *Factor V* or *Ac-globulin* (Ac = accelerator). Like prothrombin, it is a protein and is inactivated at 58 to 60° C. Unlike prothrombin it is not adsorbed by $Ca_3(PO_4)_2$ nor is its concentration in the blood diminished by vitamin K deficiency or by dicoumarol. The concentration of labile factor is relatively low in human blood as compared with rabbit blood, which contains 50 times more; yet a significant diminution of this agent rarely occurs clinically.

CALCIUM. Like prothrombin and labile factor, calcium reacts stoichiometrically in the formation of thrombin. The minimal requirement for optimum clotting of human plasma is 0.0012 M. Since the level of calcium in the blood at which fatal tetany occurs is still entirely adequate for normal clotting, hypocalcemia as a cause of bleeding is highly improbable.

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[13] Quick, Murat, Hussey, and Burgess: *Surg. Gynecol. and Obst.*, **95**, 671 (1952).

Prothrombin, labile factor, and calcium may for convenience be considered to constitute the prothrombin complex. The interrelation of these factors is stoichiometric, and since labile factor and calcium are normally in excess, prothrombin is the limiting factor and therefore the determinant of the prothrombin time except in very rare pathological states.

THROMBOPLASTINOGEN. Plasma contains a protein, closely associated in physical properties with fibrinogen and globulin, which reacts with a platelet factor to form thromboplastin. This agent, which has been named *thromboplastinogen*, is deficient in hemophilia. It is probable therefore that it is the same or closely related to the substance which some investigators call *antihemophilic globulin.*

Curiously, no preformed thromboplastin is found in the blood. Nevertheless, nearly all other tissues of the body contain this agent, and in some organs such as the lungs, brain, and placenta, it is present in relatively high concentration.

PLATELETS. These cells contain a factor which reacts with thromboplastinogen to form thromboplastin. The clotting agent occurs in the portion of the platelet called the granulomere. It is heat-stable and is probably a lipide with a high molecular weight. In addition to the participation of platelets in the formation of thrombin, they play an important role in clot retraction. Intact platelets adhere to the shafts of fibrin and, probably as the result of morphological alterations, cause a twisting, shortening, and bending of fibrin strands that results in a condensation of the fibrin mass. Platelets have a third function in hemostasis. Lysis of these cells is accompanied by the appearance in the serum of a vasoconstrictor called *serotonin* or *thrombotonin*, which is chemically 5-hydroxytryptamine. It is probable that this compound exercises an important function in stopping the flow of blood in hemorrhage.

THE CLOTTING REACTION. The basic reason why circulating blood remains fluid is that it contains no active thromboplastin. For this substance to form, platelets must interact with thromboplastinogen, but before even this can occur, thromboplastinogen must be activated by means of thrombin. The probable sequence of reactions is:

(1) thromboplastinogen $\xrightarrow{\text{thrombin}}$ thromboplastinogen A (activated)
(2) thromboplastinogen A + platelet factor = thromboplastin
(3) thromboplastin + labile factor + calcium + prothrombin = thrombin
(4) fibrinogen $\xrightarrow{\text{thrombin}}$ fibrin

Thrombin has a dual function: it clots fibrinogen and it activates thromboplastinogen. Through the latter it initiates a chain reaction, for as it activates thromboplastinogen, more thrombin is formed, accelerating the cycle of reactions. A break or injury of the vessel wall may liberate enough tissue thromboplastin to produce the initial thrombin that starts the clotting reaction. The prompt removal of thrombin is therefore necessary to safeguard against uncontrolled and massive intravascular clotting. Experimental evidence has been obtained indicating that the physiological antithrombin is fibrin.

Owing to the ability of fibrin to adsorb thrombin with avidity and because of the tremendous surface it presents to the serum dispersed in the reticulum of the clot, the removal of thrombin by fibrin is exceedingly efficient and the chain reaction is effectively held in abeyance. Since the intimate contact between serum and fibrin surface is lost when clot retraction occurs, it is very likely that this process may play a significant role in intravascular clotting.

There is increasing evidence that the clotting reactions in addition to their function in hemostasis may perhaps participate in immunological and other defense mechanisms. It is probably not accidental that thromboplastin is widely distributed in various tissues and that the daily turnover of prothrombin is nearly equal to the amount in circulation. As the physiological significance of blood clotting becomes better understood, a number of factors directly or indirectly associated with these reactions may be better evaluated. Among such factors is the antithrombin of the serum. This agent, which occurs in the albumin fraction, neutralizes large quantities of thrombin, but its action is so slow that it does not interfere with the conversion of fibrinogen to fibrin and therefore has no true anticoagulant action.

Fibrinolysin is an enzyme which dissolves fibrin. It is present in the blood as a zymogen, profibrinolysin. Interestingly, certain bacteria produce products which can activate profibrinolysin. Such activators are streptokinase and staphylokinase.

Another factor which markedly influences the clotting of blood is *heparin*, a mucopolysaccharide containing mucoitin-sulfuric acid. Heparin is present in the mast cells of Ehrlich as polychromatic-staining granules. The amount occurring in the blood is extremely small, but in anaphylactic shock its concentration may become sufficiently high to make the blood incoagulable. Heparin itself has little anticoagulant action, but it forms a powerful antithrombin with a cofactor which is present in the albumin fraction of serum.

Medicolegal Tests for Blood. The analysis of a specimen for the presence of blood may be of utmost significance. Often it is even more important to determine whether the blood is of human origin. To establish the presence of blood, a portion of the material under investigation should be extracted with 0.9 per cent sodium chloride solution and examined under the microscope. If the material contains relatively fresh blood, erythrocytes may be found and identified. An aqueous extract should be examined spectroscopically or, better, extracted with acid alcohol and examined with the spectroscope for absorption bands of acid hematin. The preparation and identification of hemin crystals is a satisfactory way to detect blood. Even old blood will readily yield these dark-brown or chocolate crystals, provided the specimen has not been exposed to a high temperature or to direct sunlight for a long period. If the amount of blood is very small, detection by the guaiac test or the benzidine reaction is of great practical usefulness. These tests are exceedingly delicate and reliable, but it is essential that the reagents be properly prepared and tested on control solutions of highly diluted blood.

After the presence of blood is established by these tests, the final step is its identification as to species origin. This can be done only by immunological procedures of which the precipitin test is the most satisfactory. This test is founded on the observation that when serum of one animal is injected into an animal of a different species, the latter will develop in its serum an agent which will react with the proteins of the foreign serum and cause the formation of a precipitate. The precipitin test is highly delicate. It will detect blood in high dilution. It is likewise very specific, since overlapping occurs only in very closely related species.

Lymph. Lymph may be considered the middleman in the transactions between blood and tissues. It is the medium by which the nutritive material and oxygen transported by the blood for the tissues are brought into intimate contact with those tissues and thus utilized. In the further fulfillment of its function, the lymph bears from the tissues water, salts, and the products of the activity and catabolism of the tissues, and passes these into the blood. Lymph therefore exercises the function of a go-between for blood and tissues. It bathes every active tissue of the animal body, and is believed to have its origin partly in the blood and partly in the tissues.

In chemical characteristics, lymph resembles blood plasma. In fact, it has been termed "blood without its red corpuscles." Lymph from the thoracic duct of a fasting animal or from a large lymphatic vessel of a well-nourished animal is of a variable color (colorless, yellowish, or slightly reddish) and alkaline in reaction to litmus. It contains fibrinogen, prothrombin, and leukocytes, and coagulates slowly, the clot being less firm and bulky than the blood clot. Serum albumin and serum globulin are both present in lymph, the albumin predominating in a ratio of about 3 or 4:1. The principal inorganic salts are sodium salts (chloride and bicarbonate); the phosphates of potassium, calcium, magnesium, and iron are present in smaller amount.

Substances which stimulate the flow of lymph are termed lymphagogues. Such substances as sugar, urea, certain salts (especially sodium chloride), peptone, egg albumin, extracts of dog's liver and intestine, crab muscles, and blood leeches are included in this class.

In a fasting animal, the lymph coming from the intestine is a clear, transparent fluid possessing the characteristics already outlined. After a meal containing fat has been ingested, this intestinal lymph is white or milky. This is termed *chyle*, and is essentially lymph possessing an abnormally high (5 to 15 per cent) content of emulsified fat. This chyle is absorbed by the lacteals of the intestine and transported to the lower portion of the thoracic duct. Apart from the fat content, the composition of lymph and chyle are similar.

Cerebrospinal Fluid. On tapping the spinal canal a water-clear fluid is obtained which is essentially a plasma filtrate formed by the choroid plexus. This fluid fills the two lateral and the third and fourth ventricles, the subarachnoid, and the spinal canal. Spinal fluid usually contains less than 50 mg. of protein per 100 ml., of which 80 per cent is albumin, the remainder globulin.

The osmotic pressure and the pH of the spinal fluid are the same as that of the plasma, whereas the glucose and calcium contents are roughly 50 per cent as great. The sodium chloride concentration is distinctly higher than that of plasma, while the bicarbonate concentration is about the same.

The analysis of the cerebrospinal fluid supplies valuable diagnostic information in the study of diseases involving the central nervous system. In general, the quantitative methods developed for plasma can be applied with minor modifications in the study of spinal fluid.

The *colloidal gold test* of Lange may also be used. In this test a colloidal gold solution is mixed with progressively increasing dilutions of cerebrospinal fluid. The orange-red color of Lange's solution is changed when cerebrospinal fluid from certain pathological conditions is tested.

EXPERIMENTS ON BLOOD

IDENTIFICATION OF BLOOD

1. *Microscopical Examination*. Place a drop of diluted blood on a microscopical slide and cover with a coverglass. Examine under a microscope.

2. *Tests for Various Constituents*. To 50 ml. of water in a large casserole, add 2 drops of acetic acid and bring to a boil. At the point of vigorous boiling, add 10 ml. of defibrinated blood slowly from a pipet, with stirring. Continue boiling and stirring for one minute after all the blood has been added. Then pour the mixture immediately onto a large folded filter which has been prepared beforehand. If the filtrate is not practically clear and colorless it should be discarded and the process repeated with more blood. Reserve the coagulum for further use. Why is the coagulum colored dark brown? Evaporate the filtrate to about 25 ml., filtering off any precipitate which may form in the process. Make the following tests upon the filtrate:

(a) BENEDICT'S TEST. **To 5 ml. of Benedict's solution add 5 drops of neutralized filtrate and boil one minute. Explain.**

(b) CHLORIDES. **To a small amount of the filtrate in a test tube add a few drops of nitric acid and a little silver nitrate. In the presence of chloride, a white precipitate of silver chloride will form.**

(c) PHOSPHATES. **Test for phosphates by nitric acid and molybdate solution according to directions given on p. 213.**

(d) CALCIUM. **To 5 ml. of filtrate add 0.1 ml. of 4 per cent ammonium oxalate. Calcium oxalate precipitates as fine crystals. For crystalline form see p. 855.**

(e) TEST FOR IRON. **Incinerate a small portion of the coagulum obtained above, in a porcelain crucible. Cool, dissolve the residue in dilute hydrochloric acid, and test for iron by potassium ferrocyanide or ammonium thiocyanate. Which of the constituents of the blood contains the iron?**

(f) CRYSTALLIZATION OF SODIUM CHLORIDE. **Place the remainder of the filtrate in a watch glass and evaporate it on a water bath. Examine the crystals under the microscope and compare them with those in Fig. 112.**

3. *Hemin Tests*. Hemoglobin is readily split into its globin and hemin components by means of heat and acetic acid. Hemin forms characteristic crystals which are readily recognized under the microscope and serve as a reliable test for blood.

(a) TEICHMANN'S METHOD. Place a very small drop of blood on a microscopical slide, add a small drop of water, and stir to lake the blood. Add a fraction of a drop of dilute (0.9 per cent) sodium chloride or potassium chloride solution and carefully evaporate to dryness over a low flame. Put a coverglass in place, run underneath it a drop of glacial acetic acid, and warm gently until the formation of gas bubbles is noted. Add another drop of glacial acetic acid, cool the preparation, examine under the microscope, and compare the crystals with those shown in Fig. 113.

(b) NIPPE'S METHOD. Spread a small drop of blood on a slide in the form of a film and evaporate to dryness over a low flame. Now add 2 drops of a solution containing 0.1 g. each of potassium chloride, iodide, and bromide in 100 ml. of glacial acetic acid. Place a coverglass in position and heat gently over a low flame until gas bubbles form and the solution boils. Run 1 to 2 drops of the reagent underneath the coverglass and examine under a microscope. Compare the crystals with those shown in Fig. 113.

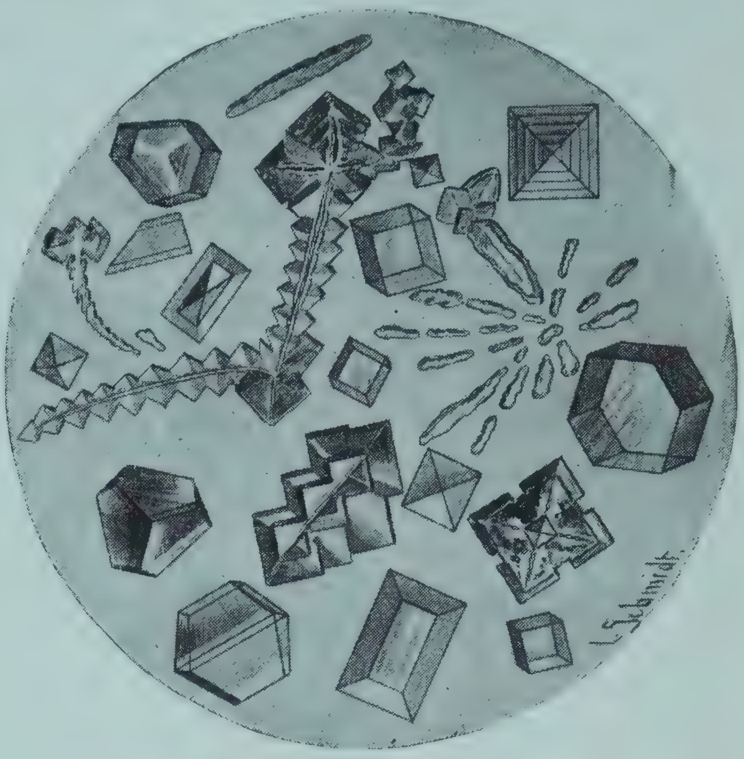

FIG. 112. SODIUM CHLORIDE.

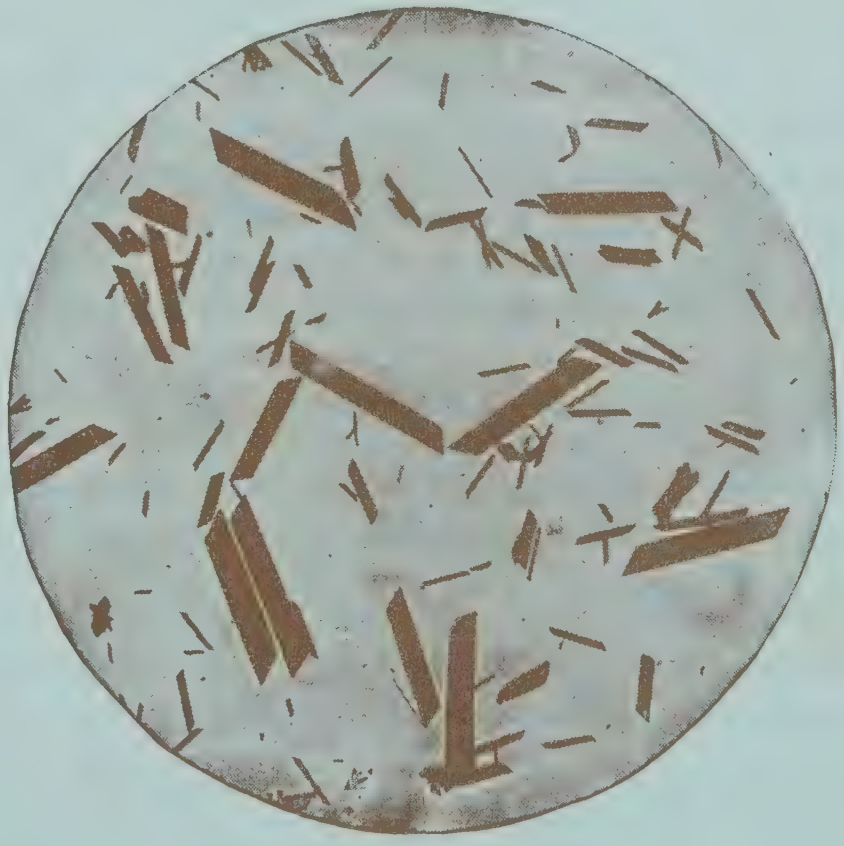

FIG. 113. HEMIN CRYSTALS FROM SHEEP BLOOD.

Reproduced from a photomicrograph furnished by the late Prof. E. T. Reichert, of the University of Pennsylvania.

This method is more rapid than Teichmann's method and crystals of inorganic chlorides are not formed. In Teichmann's method crystals of sodium chloride (Fig. 112) often obscure the hemin crystals. For the preparation and purification of hemin on a large scale, see *Organic Syntheses*, 21, 53, New York, John Wiley & Sons, Inc., 1941.

4. ***Guaiac Test.*** This method is sensitive and valuable for detecting blood. Care must be taken not to use too concentrated a solution of guaiac since a voluminous precipitation of resinous material may easily obscure the blue color. Substances other than blood, such as milk, pus, and saliva, may give a positive test, but after boiling for 15 to 20 seconds they no longer yield a blue color with guaiac, whereas blood similarly subjected to boiling still yields a positive test.

The test follows: By means of a pipet drop a solution of guaiac in glacial acetic acid (strength about 1:60) into the solution under examination[14] until a turbidity is observed, and add hydrogen peroxide, drop by drop, until a blue color is obtained.

5. ***Benzidine Reaction.*** This test is one of the most delicate for detecting blood, but care must be taken to use a good grade of benzidine, since the sensitivity is greatly influenced by the purity of the reagent. The test depends on the action of hemoglobin, which catalytically decomposes hydrogen peroxide, thereby liberating oxygen which oxidizes benzidine to a blue or green derivative. Since an excess of hydrogen peroxide interferes with the reaction, it is essential that the details of the procedure be followed scrupulously. It is particularly important that the peroxide be added last. The benzidine solution is unstable, especially when exposed to light. It should therefore be prepared fresh daily and kept in a brown bottle or a dark place. The test is particularly valuable to detect occult blood in the feces.[15]

The test is performed as follows: To 3 ml. of a saturated solution of benzidine in glacial acetic acid[16] add 2 ml. of the solution to be tested and 1 ml. of 3 per cent hydrogen peroxide. A positive test is indicated by a blue or green color. The following modification is a much more delicate and reliable test.

Confirmatory Test. **Make 10 ml. of the solution acid with acetic acid and extract by shaking with 5 ml. of ether. The acid breaks up the hemoglobin to globin and heme and the latter is extracted by the ether. Pour off the ether into a small evaporating dish. Put on a hot water bath (with the flame turned out). Evaporate to dryness. To the residue add a few drops of water, a drop of saturated solution of benzidine in glacial acetic acid, and a drop of 3 per cent hydrogen peroxide. A blue or green color indicates blood.**

Lyle, Curtman, and Marshal Modification. **Into a perfectly clean dry test tube introduce 1.4 ml. of benzidine solution,[17] add 0.2 ml. of water or glacial acetic acid, then 1 ml. of the fluid to be tested and finally 0.4 ml. of 3 per cent hydrogen peroxide. Note the appearance of a blue color, which reaches its maximum in 5 to 6 minutes.**

6. ***Hemochromogen Test.*** **Add 2 or 3 drops of Takayama's solution[18] to a small piece of suspected material on a slide. Cover with a coverglass. Examine**

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[14] Alkaline solutions should be made slightly acid with acetic acid, since the blue end reaction is very sensitive to alkali. This is particularly necessary if an alcoholic solution of guaiac is used.

[15] Hughes: *Brit. Med. J.*, 2, 970 (1952).

[16] Glacial acetic acid is preferable, but if it is not available, alcohol acidified with acetic acid may be used.

[17] Benzidine solution may be prepared as follows: Place 4.33 ml. of glacial acetic acid in a small Erlenmeyer flask, warm to 50°, and add 0.5 g. of benzidine. Heat the flask for eight to ten minutes in water at 50°. To the resultant solution add 19 ml. of distilled water. This solution may be kept for several days without deterioration.

[18] A mixture of 3 ml. of 10 per cent NaOH, 3 ml. of pyridine, 3 ml. of a saturated solution of glucose, and 7 ml. of water. The solution works rapidly in the cold if at least 24 hours old. With a fresh solution, warming or more time is necessary. It keeps for from one to two months.

under the microscope. Salmon pink crystals should appear in 1 to 6 minutes. At the same time the color changes through green-brown and dark red to pink, indicating the formation of a hemochromogen and confirming the test. The crystals have a shallow rhomboid form (see Fig. 114).

This method is simpler to use than the hemin test and may be used to confirm the latter in doubtful cases. It is not always given by old blood stains (over six months old) and hence does not replace the hemin test. The glucose may act as a reducing agent as well as by decreasing the solubility of the hemochromogen. Schumm has cast some doubt on the specificity of this test for blood.

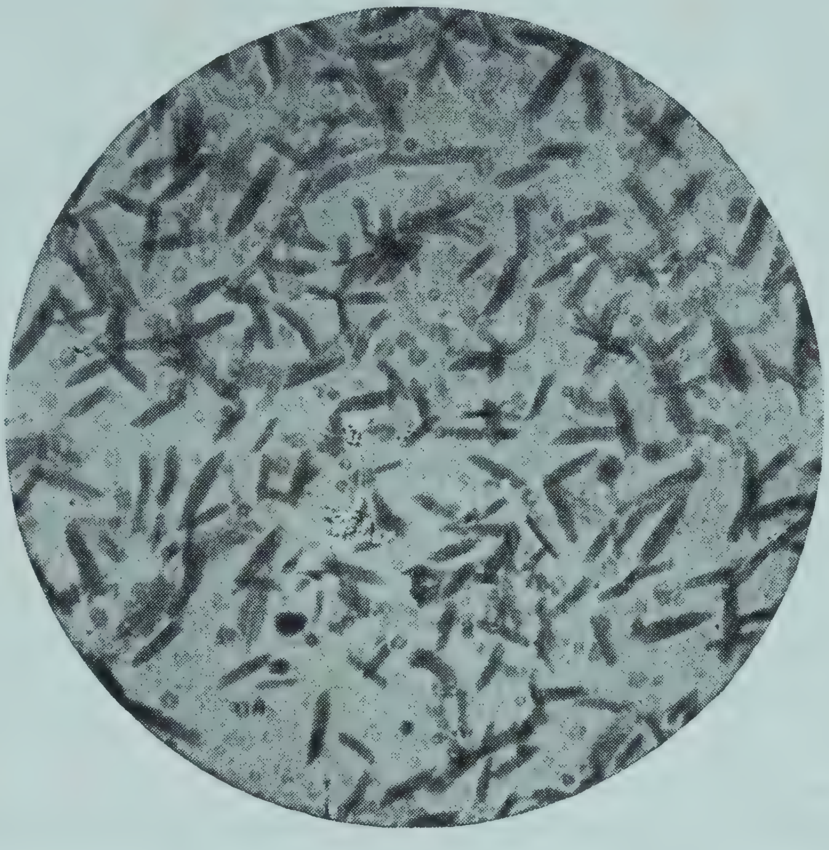

FIG. 114. HEMOCHROMOGEN CRYSTALS.
Prepared and photographed by Dr. Arthur G. Cole, University of Illinois, College of Medicine.

7. Immunological Determination of Blood Species. The serum proteins give immunological reactions, such as the precipitin reaction, which are specific not only for the individual proteins of the serum but also for the species of animal from which the serum is obtained. These immunological reactions are therefore used in medicolegal investigations to determine the species to which a particular blood sample or stain belongs. The species specificity of these reactions is demonstrated in the following experiments on the precipitin reactions of dog, beef, and human serums. Antiserums against human blood react with the serums of some of the higher apes, indicating a close relationship between the various species. Usually, however, even these reactions show quantitative differences which are sufficient to differentiate between human and other bloods.

(a) PREPARATION OF IMMUNE SERUMS (ANTISERUMS). **Prepare approximately 1 per cent solutions of dog, beef, and human serum proteins by diluting 15 ml. of the clear serums to 100 ml. with physiological (0.9 per cent) salt solution. Immunize rabbits against each of these solutions (antigens) by injection of the diluted serums into the marginal ear vein. Make the first injection with 1 ml. of antigen and follow with injections of 2, 4, 6, and 8 ml. portions at intervals of three or four days. On the fourth day after the last injection, collect 1 ml. of blood and test the serum for precipitin content by the method** described below. If the precipitin is present in sufficiently high titer, bleed the rabbit from the heart, allowing the blood to coagulate. Transfer the clear serum to sterile vials closed with rubber stoppers. Add a few drops of chloroform to each vial as a preservative, and keep vials in a refrigerator when not in use.

An alternative method for preparing the antiserums is as follows: Twenty-five ml. of serum is diluted with 80 ml. of distilled water, and to this solution 90 ml. of 10 per cent potassium alum is added. The pH is adjusted to the isoelectric point specific to the alum-protein complex (human serum, pH 6.5) with 5 N NaOH. The precipitate is washed twice with 200 ml. saline containing 1:10,000 merthiolate, and then is suspended in 100 ml. of saline (1:10,000 merthiolate).

The immunizing dose is 10 ml. of the suspension, which is injected intramuscularly—5 ml. into each hind leg of a rabbit. After 15 to 20 days the rabbit is taken off food for 24 hours and then bled. The serum obtained is tested for specificity and sensitivity. For a positive result a precipitin at a dilution of a 1:100 antigen should be obtained within five minutes. As a control, an antigen of a species to which the rabbit is not sensitized should show no precipitin in a dilution of 1:50.

(b) TITRATION OF ANTISERUMS. Prepare the following dilutions of the antigen (diluted serum or protein solution to be tested) using physiological saline as diluent: 1:100; 1:1,000; 1:10,000; 1:50,000. Transfer 1 ml. of each dilution to a separate small test tube which must be scrupulously clean and dry. With a capillary pipet carefully introduce 0.2 ml. of the immune serum at the bottom of each tube so as to stratify the antigen solution above the serum. With a 1:1,000 dilution, a white ring or cloud will form at the interface in a few minutes if the test is positive.

For control, place in one test tube 1 ml. of a 1:50 dilution of an antigen against which the rabbit was not immunized, and add 0.1 ml. of the antiserum as in the test above. For a second control, transfer 1 ml. of the 1:1,000 dilution of the antigen to a test tube and add 0.1 ml. of normal rabbit serum. No precipitation should form in either tube.

(c) IMMUNOLOGICAL EXAMINATION OF BLOOD STAINS. Extract the stain with 1 or 2 ml. of physiological salt solution. Filter and use the filtrate for making up dilutions of antigen and for precipitin reactions, as described above.

*8. **Hemagglutination.*** The common garden bean, such as the scarlet runner,[19] contains a protein substance which exhibits the interesting property of causing clumping or agglutination of red blood corpuscles.

Dilute defibrinated blood[20] ten times with physiological sodium chloride solution (0.9 per cent) and place 1 ml. in each of three small test tubes.

Grind 3 beans to a fine meal in a coffee mill or with mortar and pestle and extract for a few minutes with 0.9 per cent sodium chloride solution. Filter and add 0.05 ml. (about 2 to 3 drops) of the filtered extract to the first of the blood tubes, 0.01 ml. to the second, and 0.05 ml. of 0.9 per cent sodium chloride to the third.

Invert each tube to mix the contents thoroughly, and note the rapid agglutination and precipitation of the blood corpuscles in the first tube, a less rapid agglutination in the second, while the third or control tube remains unaltered. In half an hour the corpuscles in the first tube often are packed solid and one is able to pour off perfectly clear serum.

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[19] The scarlet runner is a familiar variety purchasable in every seed store. It occurs in two varieties, the *white* and the *red*. Ricin, a protein constituent of the castor bean, also possesses pronounced agglutinating properties. Because of its poisonous nature it is, however, not suitable for use in class experiments.

[20] Rabbit's blood is especially desirable and may be obtained for the purpose by bleeding from a small cut on the animal's ear and defibrinating.

If the remainder of the bean extract is boiled for a few minutes, the coagulum filtered out and 0.05 ml. of the filtrate added to the control tube, no agglutination occurs, indicating that the hemagglutinin has been destroyed or removed by the boiling.

9. Red Cell Fragility. When red cells are placed in a hypotonic saline solution, water passes into the cell until the osmotic pressure within and without are equalized. This brings about a swelling of the red cell which if sufficiently great causes the cell membrane to rupture, thereby resulting in hemolysis. Normal erythrocytes remain intact in as low as 0.44 per cent saline, whereas defective red cells, present in certain types of disease such as hereditary hemolytic jaundice, show beginning hemolysis at this concentration and complete hemolysis at 0.34 per cent.

Procedure. Prepare a 1 per cent solution of sodium chloride and from this make a series of dilutions ranging from 0.30 to 0.58 per cent by placing 0.60 ml. in the first test tube and 0.64 ml. in the second, increasing each successive tube by 0.04 ml. until the fifteenth, to which 1.16 ml. is added. Add distilled water to each tube to bring the volume to 2 ml. To each tube add one drop of blood obtained by venipuncture. Mix and allow to stand at room temperature for 2 hours. Record as beginning hemolysis the tube showing a slight tinge of red in the supernatant, and as complete hemolysis, the tube in which all cells have disappeared.

10. Crystallization of Oxyhemoglobin (Reichert's Method). Add to 5 ml. of the blood of the dog, horse, guinea pig, or rat, before or after laking or defibrinating, from 1 to 5 per cent of ammonium oxalate in substance. Place a drop of this oxalated blood on a slide and examine under the microscope. The crystals of oxyhemoglobin will be seen to form at once near the margin of the drop, and in a few minutes the entire drop may be a solid mass of crystals. Compare the crystals with those shown in Figs. 107 and 108, p. 472.

In some species (e.g., the rat) oxyhemoglobin tends to crystallize out of blood very rapidly. Merely pressing a small drop of blood between a coverglass and a microscope slide will result in a mass of oxyhemoglobin crystals.

11. Preparation of Hemoglobin (Method of Marshall and Welker). Draw blood into a flask and defibrinate by shaking with glass beads. Strain through cheesecloth. Centrifuge. Wash corpuscles three or four times with 0.9 per cent NaCl. Add ether a few drops at a time with thorough mixing until a clear solution is obtained. If the solution is viscid add a little water and then an equal volume of aluminum hydroxide cream (see Appendix). Mix thoroughly and filter. Cool to 0° C. and add absolute alcohol (also cooled to 0°) to make the alcohol percentage 20 to 30 per cent. Let stand at a few degrees below 0° C. Wash the crystals by decantation with 25 per cent alcohol at 0° C. Dry in a desiccator over sulfuric acid.

12. Demonstrations of Hemolysis and Osmotic Pressure.

(a) HEMOLYSIS (LAKING BLOOD). Note the opacity of ordinary defibrinated blood. Place a few ml. of this blood in a test tube and add water, a little at a time, until the blood is rendered transparent. Hemolysis has taken place. How does the water act in causing this transparency? Examine a drop of

hemolyzed blood under the microscope. How does its microscopical appearance differ from that of unaltered blood? What other agents may be used to bring about hemolysis?

(b) OSMOTIC PRESSURE. **Place a few ml. of blood in each of three test tubes. Hemolyze the blood in the first tube according to directions given in (a), above. Add an equal volume of isotonic (0.9 per cent) sodium chloride to the blood in the second tube, and an equal volume of 10 per cent sodium chloride to the blood in the third tube. Mix thoroughly by shaking, and after a few moments examine a drop from each of the three tubes under the microscope (see Figs. 115 and 116). What do you find and what is your explanation from the standpoint of osmotic pressure?**

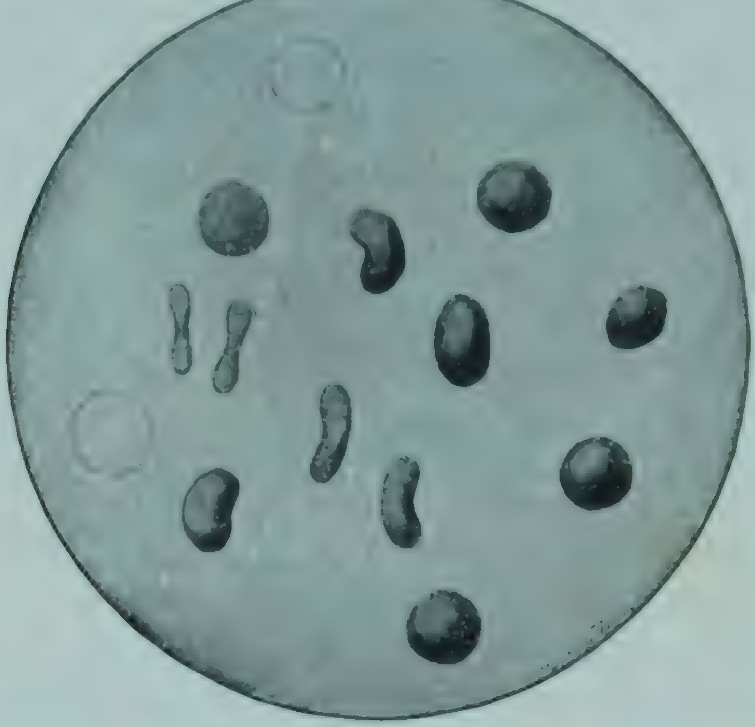

FIG. 115. EFFECT OF WATER ON ERYTHROCYTES.

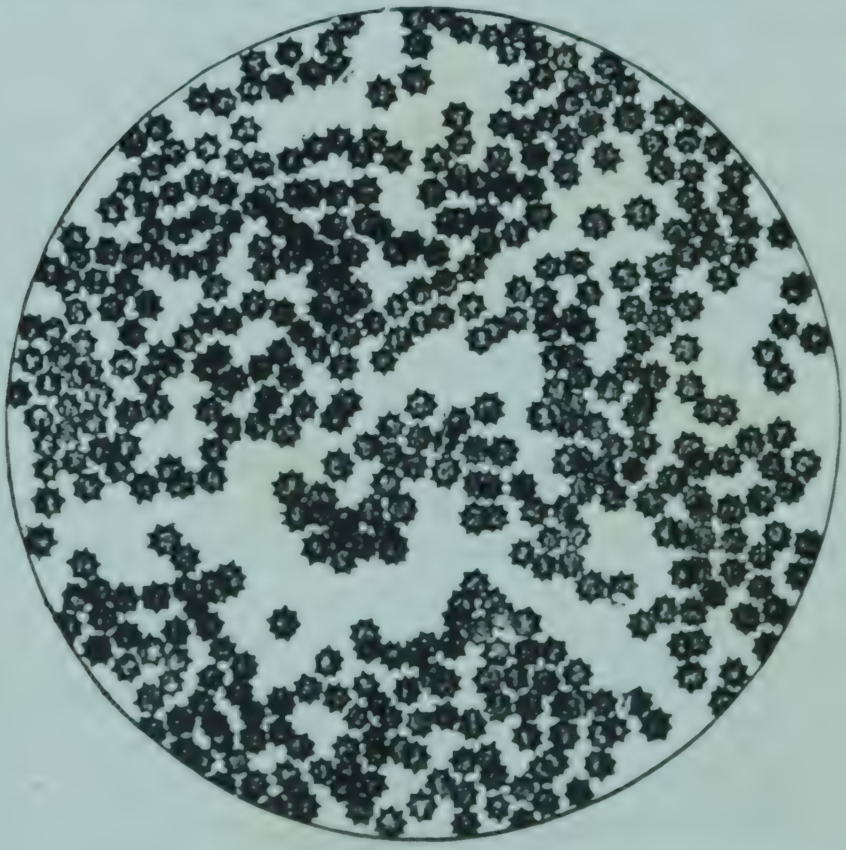

FIG. 116. CRENATED ERYTHROCYTES.

PLASMA AND SERUM

Plasma is the noncellular fraction of the blood. It is conveniently obtained by centrifugation. If no anticoagulant is used, the plasma can be designated as native. To obtain such plasma, it is best to collect the blood with a silicone-coated syringe and needle[21] and to transfer it to a tube similarly coated. Usually sodium citrate or oxalate is mixed with the blood to make it incoagulable. It is customary to name the plasma according to the anticoagulant employed, as for instance, oxalated, citrated, or heparin plasma.

Serum is the fluid obtained after blood has clotted. It has essentially the same composition as plasma except that it lacks fibrinogen and has a diminished concentration of various clotting factors.

1. *Preparation of Oxalated Plasma.* Collect 9 ml. of blood by venipuncture and mix immediately with 1 ml. of 0.1 M sodium oxalate[22] in a test tube or centrifuge tube. Centrifuge at 2500 r.p.m. for 5 minutes. Remove the plasma with a pipet. By attaching the rubber bulb of a medicine dropper to the pipet, the plasma can readily be drawn off.

2. *Preparation of Serum.* Place approximately 5 ml. of freshly drawn blood in a small test tube. Put the tube in a water bath at 37° C. for 30 minutes and then centrifuge at 2500 r.p.m. for 10 minutes.

3. *Separation of Fibrinogen, Globulin, and Albumin.* Add 0.5 ml. of half-saturated ammonium sulfate[23] to 0.5 ml. of oxalated plasma. Note the amount of precipitated fibrinogen formed. Repeat adding 0.5 ml. of saturated sodium chloride[24] to 0.5 ml. of oxalated plasma. Compare the amounts of fibrinogen obtained by the two salting-out methods.

Repeat the experiment, adding half-saturated ammonium sulfate and saturated sodium chloride to equal volumes of serum. Why do the solutions remain clear?

To 0.5 ml. of serum add 0.5 ml. saturated ammonium sulfate and mix. A precipitate of globulin is formed. Remove the salted-out protein either by centrifugation or filtration. To the clear filtrate, add small quantities of powdered ammonium sulfate and note the formation of a precipitate, which is albumin.

Outline a scheme of separating fibrinogen, globulin, and albumin.

Test samples of the precipitated fibrinogen, globulin, and albumin with the biuret, Millon's, and Hopkins-Cole tests.

4. *Heat Coagulation of Plasma Proteins.* Transfer 0.5 ml. of plasma and 0.5 ml. of serum to separate tubes and place in a water bath at 60° C. Examine after 10 minutes. Serum remains clear because it does not contain fibrinogen. Gradually increase the temperature of the bath and note when heat coagulation appears in the serum.

---

[21] *Coating glass with silicone.* Dilute one volume of Silicone (methyl-chlorosilane-Dri-Film N 9987, General Electric) with 3 volumes of toluene. Thoroughly clean the glassware and dry. Cover the surface to be coated with the silicone solution. Pour off the excess and drain for 30 minutes. Rinse in warm running water and dry. To obtain a thorough coating, polish the surface with a nylon cloth and recoat several times.

[22] *Sodium oxalate, 0.1 M.* Dissolve 1.34 g. sodium oxalate (*c.p.*) in 100 ml. of distilled water.

[23] *Saturated ammonium sulfate.* Cover 90 g. of ammonium sulfate (*c.p.*) with 100 ml. of distilled water. Mix and allow to stand for 24 hours. The supernatant liquid is saturated. Dilute with distilled water to obtain any desired saturation.

[24] *Saturated sodium chloride.* Cover 40 g. with 100 ml. of distilled water, mix, and allow to stand 24 hours.

BLOOD COAGULATION

1. Clotting Time of Recalcified Plasma. On adding calcium to citrated or oxalated plasma, clotting will occur. The probable over-all equation to express the reaction is:

thromboplastin + labile factor + calcium + prothrombin = thrombin

How these factors interact is not known, but there is strong evidence that the composite reaction is essentially stoichiometric. The thromboplastin does not exist preformed in the blood but is the product of the interaction of a platelet constituent and thromboplastinogen. The relatively slow and small production of thromboplastin is the limiting factor in the reaction and is therefore the determinant of the clotting time of recalcified plasma. If blood is oxalated in a test tube coated with material giving a nonwettable surface such as paraffin or silicone, the platelets are preserved and can be removed by high centrifugation. Plasma thus obtained clots much more slowly on recalcification.

Procedure. Transfer 0.1 ml. of oxalated plasma to a small test tube and place in a water bath at 37° C. Blow 0.2 ml. 0.01 M calcium chloride[25] into the plasma to attain instantaneous mixing, and time the clotting exactly with a stop watch. Examine the tube every 15 seconds by gently tilting to detect the exact moment the incipient clot appears. Normal human oxalated plasma collected in glass clots in 90 seconds to 120 seconds after recalcification.

To determine the effect of the removal of platelets on the clotting time, collect 10 ml. of blood by venipuncture. Transfer 0.5 ml. of 0.1 M sodium oxalate to a glass test tube and the same quantity to a test tube coated with paraffin. Add 4.5 ml. of blood to each tube and mix by inverting the tube after covering it with wax paper. Chill the blood by placing it in an ice bath for 10 minutes. Centrifuge the paraffin-coated test tube at 2,000 r.p.m. for 15 minutes, and the glass test tube at the same speed but only for 3 minutes. Determine the clotting time of both plasmas on recalcification by the method as outlined.

2. Prothrombin Time. The relative slowness with which oxalated plasma clots after recalcification is due to the small amount of available thromboplastin. By adding an excess quantity of a potent standardized thromboplastin reagent[26] such as is readily prepared from rabbit brain, and a fixed amount of calcium, the clotting time can be made a quantitative measure of the prothrombin concentration, since the calcium and thromboplastin are constants in the reaction.

Procedure. Transfer 0.1 ml. of oxalated plasma to a small test tube, add 0.1 ml. of thromboplastin reagent, and place in a water bath kept at 37° C. Blow 0.1 ml. of 0.01 M calcium chloride measured in a short pipet into the

---

[25] *Calcium chloride, 0.01 M.* Dissolve 0.11 g. in 100 ml. of distilled water.

[26] *Preparation of Thromboplastin Reagent.* Remove the brain of a freshly killed rabbit and clear of all visible blood vessels. Triturate the material in a mortar with 20 ml. acetone and 0.1 ml. 0.2 *M* sodium citrate to remove traces of calcium. Avoid vigorous grinding until the material becomes flaky. Pour off the spent acetone. Replace with 20 ml. of a fresh portion and again triturate. Repeat until the material is granular and nonadhesive. Filter by suction and dry at 37° C. for 30 minutes.

Mix 200 mg. of the acetone-dehydrated brain with 5 ml. 0.85 per cent sodium chloride and incubate at 50° C. for 20 minutes. Mix occasionally by blowing through with a pipet. Place in a water bath at 37° C. and allow to settle sufficiently to permit drawing the solution into a pipet.

mixture. Click the stop watch at the moment the calcium chloride is added and time accurately the appearance of the clot. This is best done by leaving the test tube in the water bath until shortly before clotting occurs, then holding the tube towards a good light, and tilting gently to note the incipient formation of fibrin. Normal fresh oxalated human plasma has a prothrombin time of 11½ to 12½ seconds. When the plasma is mixed with saline in varying proportions, the following values are obtained:

Dilution of Plasma	*Concentration of Prothrombin*	*Prothrombin Time*
	per cent	*seconds*
0	100	12
4 to 5	80	13
3 to 5	60	14
2½ to 5	50	15
2 to 5	40	17
1½ to 5	30	19½
1 to 5	20	24 to 26
½ to 5	10	37 to 42

By means of a serological pipet graduated in 0.1 ml. prepare the above dilutions and determine the prothrombin time of each mixture.

3. *Preparation of Thrombin* (*Eagle*). Dilute 10 ml. of fresh citrated or oxalated plasma with 100 ml. of cold distilled water. Chill the mixture to about 5° C. in ice water and then bubble carbon dioxide gas through the solution for 5 to 10 minutes. Centrifuge off the precipitate and discard the supernatant fluid. Dissolve the residue in 10 ml. of 0.85 per cent sodium chloride solution and adjust the resulting solution to approximately pH 7.0 by the addition of sodium bicarbonate. Add to this final solution one-twentieth its volume of 0.1 M calcium chloride solution (1.1 per cent). Warm to 37° C. for a few minutes, insert a glass rod into the clot which forms and wrap the clot around the rod until all entrained fluid has been pressed out. Discard the clot, filter the remaining fluid, and store in the refrigerator. If kept cold, this thrombin solution is stable for about a week. Dried preparations of thrombin in sealed containers, which are stable indefinitely, may now be obtained from wholesale drug supply houses.

CLOTTING TIMES. When 0.1 ml. of this thrombin solution is added to 0.2 ml. of oxalated plasma, coagulation occurs in about 3 seconds. Prepare the following dilutions of thrombin with distilled water: 1:2, 1:5, 1:10, 1:20 and 1:40. Determine the thrombin time of each dilution as follows. Transfer 0.2 ml. of oxalated plasma to a test tube, place in a water bath at 37° C. Blow 0.1 ml. of the thrombin solution into the plasma and accurately determine the clotting time with a stop watch. To prepare a curve, plot the clotting times against the dilution of thrombin, taking the undiluted thrombin preparation as 100.

4. *Preparation of Fibrin.* Allow blood to flow directly from the animal into a vessel and rapidly whip it by means of a bundle of twigs, a mass of strong cords, or a specially constructed beater. If a pure fibrin is desired it is not best to attempt to manipulate a large volume of blood at one time. After the fibrin has been collected it should be freed from any adhering blood clots

and washed in water to remove further traces of blood. The pure product should be very light in color. It may be preserved under glycerol, dilute alcohol, or chloroform water.

(a) SOLUBILITY. **Try the solubility of small shreds of freshly prepared fibrin in water, dilute acid, and alkali.**

(b) PROTEIN COLOR TESTS. **Test a portion of fibrin by Millon's test, the Hopkins-Cole test, and the biuret reaction. What amino acids have you shown to be present in fibrin?**

SPECTROSCOPIC EXAMINATION OF BLOOD

As indicated in the text on p. 474, the spectroscope is a useful tool in the study of the blood pigments because of the differences in light absorption which these pigments show, and which are evident upon spectroscopic examination. Fig. 117 shows a diagrammatic representation of the visible spectrum as seen with a spectroscope, including the approximate location and width of the various colored zones of the spectrum. If a solution containing a colored substance is placed between the spectroscope and the source of light, one or more dark zones or bands are seen in the spectrum, corresponding to the absorption of light of certain specific wavelengths by the colored substance. It is frequently possible to distinguish between various pigments by the location and intensity of these absorption bands.

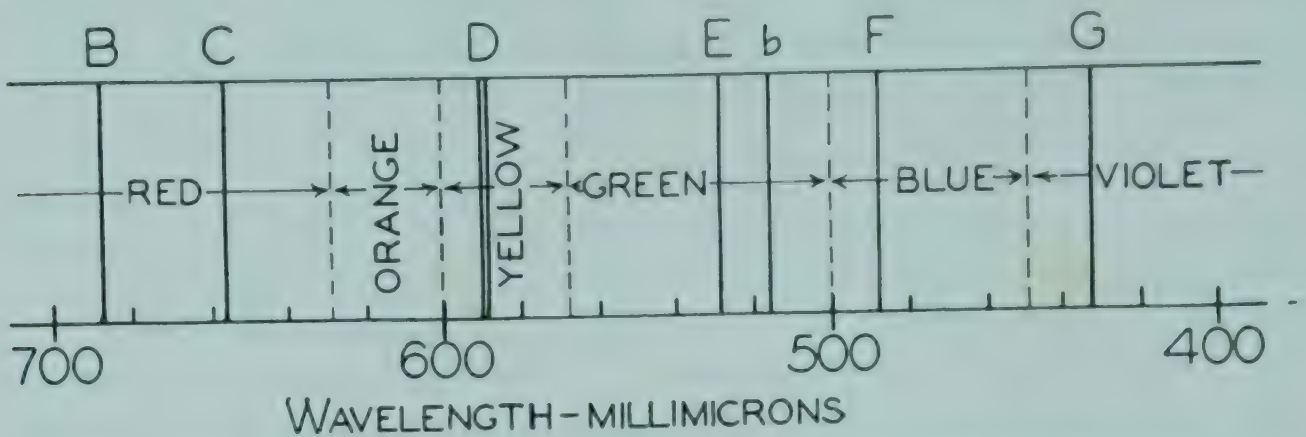

FIG. 117. DIAGRAMMATIC REPRESENTATION OF SOLAR SPECTRUM. Shown are approximate extent of colored zones and location of principal Fraunhofer lines (*B*, *C*, *D*, *E*, *b*, *F*, *G*).

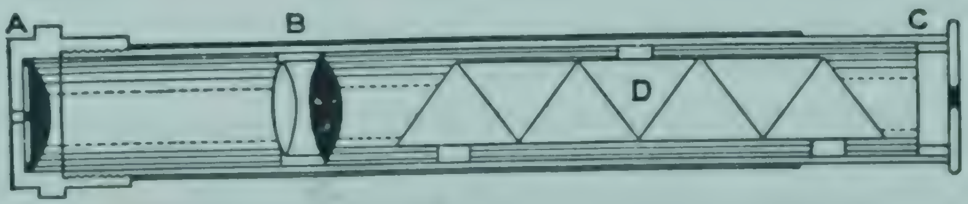

FIG. 118. DIRECT-VISION SPECTROSCOPE.

Since the ordinary hand spectroscope is not equipped with a wavelength scale, the absorption bands may be located by reference to certain of the more prominent *Fraunhofer lines* of the sun's spectrum. These are *dark* lines, readily visible in the spectrum of sunlight, which correspond to the presence of certain elements in the vapors surrounding the sun. As shown in Fig. 117, the most prominent lines and their approximate wavelengths are as follows: B, 687mμ; C, 656mμ; D, 589mμ; E, 527mμ; b, 517mμ; F, 486mμ; G, 431mμ.

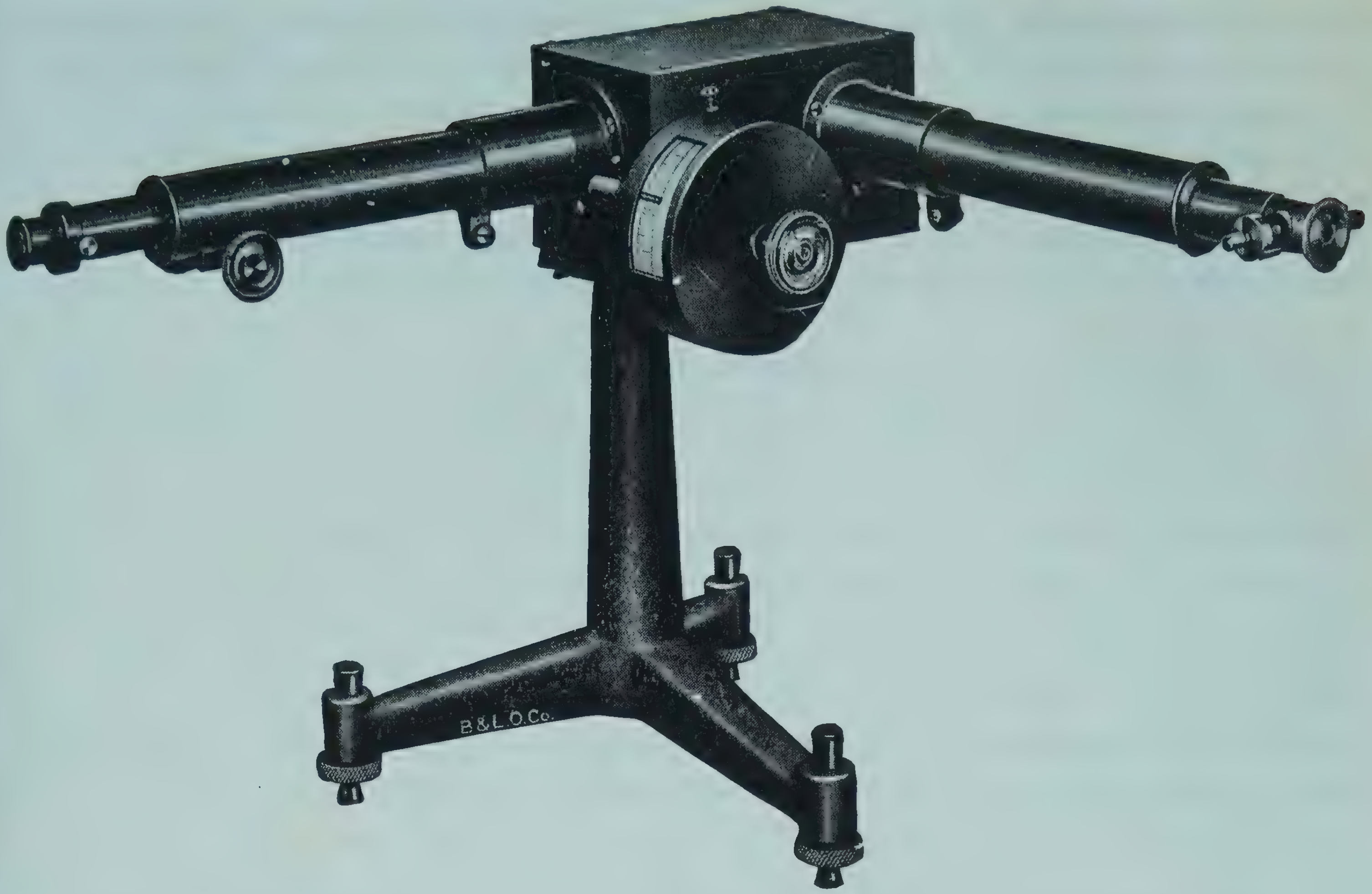

FIG. 119. ANGULAR-VISION SPECTROMETER.

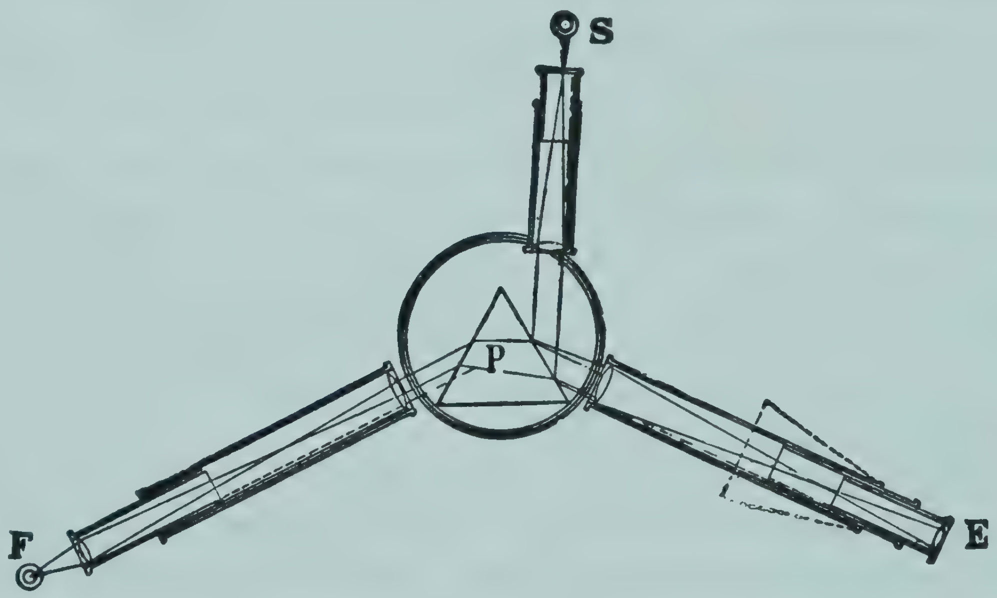

FIG. 120. DIAGRAM OF ANGULAR-VISION SPECTROSCOPE (LONG).

The white light, *F*, enters the collimator tube through a narrow slit and passes to the prism, *P*, which has the power of refracting and dispersing the light. The rays then pass to the double convex lens of the ocular tube and are deflected to the eyepiece, *E*. The dotted lines show the magnified virtual image which is formed. The third tube contains a scale whose image is reflected into the ocular and shown with the spectrum. Between the light, *F*, and the collimator slit is placed a cell to hold the solution undergoing examination.

Either the *direct-vision* spectroscope (Fig. 118) or the *angular-vision* spectroscope (Figs. 119 and 120) may be used in making the spectroscopic examination of the blood. For a complete description of these instruments the student is referred to any standard textbook of physics or to the catalogs of the manufacturers.

1. Oxyhemoglobin. Examine dilute (1:50) defibrinated blood spectroscopically. Note the broad absorption band between D and E. Continue the dilution until this single broad band gives place to two narrow bands, the one nearer the D line being the narrower. These are the typical absorption bands of oxyhemoglobin obtained from dilute solutions of blood. Now dilute the blood very freely and note that the bands gradually become narrower and, if the dilution is sufficiently great, finally disappear entirely.

2. Hemoglobin (So-called Reduced Hemoglobin). To blood which has been diluted sufficiently to show well-defined oxyhemoglobin absorption bands, add a small amount of Stokes's reagent.[27] The blood immediately changes in color from a bright red to violet-red. The oxyhemoglobin has been reduced through the action of Stokes's reagent and reduced hemoglobin has been formed, by the removal of the oxygen from the oxyhemoglobin. Examine this solution spectroscopically. Note that in place of the two absorption bands of oxyhemoglobin we now have a single broad band lying almost entirely between D and E. This is the typical spectrum of hemoglobin. If the solution showing this spectrum be shaken in the air for a few moments, it will again assume the bright red color of oxyhemoglobin and show the characteristic spectrum of that pigment.

3. Carbon Monoxide Hemoglobin. The preparation of this pigment may be easily accomplished by passing ordinary illuminating gas[28] through defibrinated ox blood. Blood thus treated assumes a brighter tint (carmine) than that imparted by oxyhemoglobin. Examine the carbon monoxide hemoglobin solution spectroscopically. Observe that the spectrum of this substance resembles the spectrum of oxyhemoglobin in showing two absorption bands between D and E. The bands of carbon monoxide hemoglobin, however, are somewhat nearer the violet end of the spectrum. Add some Stokes's reagent to the solution and again examine spectroscopically. Note that the position and intensity of the absorption bands remain unaltered.

The following are delicate chemical tests for the detection of carbon monoxide hemoglobin:

ALKALI TEST. Mix two drops of the suspected blood on a porcelain plate with an equal volume of 25 per cent NaOH. A reddish color remains in the presence of carbon monoxide hemoglobin. Treat two drops of normal blood in the same way. A brownish color is obtained.

TANNIN TEST. Divide the blood to be tested into two portions and dilute each with 4 volumes of distilled water. Place the diluted blood mixtures in two small flasks or large test tubes and add 20 drops of a 10 per cent solution of potassium ferricyanide.[29] Allow both solutions to stand for a few minutes, then stopper the vessels and shake one vigorously for 10 to 15 minutes, occasionally removing the stopper to permit air to enter the vessel.[30] Add 5 to 10 drops of ammonium sulfide (yellow) and 10 ml. of a 10 per cent solution of tannin to each flask. The contents of the shaken flask will soon exhibit the formation of a dirty olive-green precipitate, whereas the flask which was not shaken and which, therefore, still contains carbon monoxide hemoglobin will exhibit a bright red precipitate, characteristic of carbon monoxide hemoglobin. This test is more delicate than the spectroscopic test and serves to detect the presence of as low a content as 5 per cent of carbon monoxide hemoglobin.

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[27] See Appendix.

[28] The so-called water gas with which ordinary illuminating gas is diluted contains usually as much as 20 per cent of carbon monoxide (CO).

[29] This transforms the oxyhemoglobin into methemoglobin, which does not combine with carbon monoxide.

[30] This is done to dissipate any carbon monoxide present.

DILUTION TEST. In very dilute solution oxyhemoglobin appears yellowish-red, while carbon monoxide hemoglobin under the same conditions appears pinkish- or bluish-red. Dilute a drop of normal blood with water, and dilute in parallel fashion a drop of blood containing carbon monoxide hemoglobin with water, until by comparison a difference in tint is noted. This is said to be as satisfactory a test as any for routine purposes.

QUANTITATIVE DETERMINATION OF CARBON MONOXIDE.[31] If possible collect blood from an arm vein in an oxalated tube. Otherwise wrap a finger of the subject with a rubber band. Prick deeply with a blood lancet. Draw 0.1 ml. of blood into a pipet and discharge into a test tube containing 0.9 ml. of distilled water, drawing back once or twice to insure removal of adhering blood. Mix immediately but not too vigorously. Add 1 ml. of freshly prepared pyrogallic–tannic acid solution[32] and mix by inverting twice. After 15 minutes compare with standards prepared as follows: Lake 1 volume of oxalated human blood free from carbon monoxide with 9 volumes of distilled water. Saturate half of the laked blood with CO by rotating in a flask or separatory funnel filled with illuminating gas for 15 to 20 minutes, avoiding violent agitation. Mix proportional parts of the treated and untreated blood solutions to represent 0, 10, 20, 30, 40, . . . 100 per cent saturation. Transfer 1-ml. portions of these solutions to test tubes of the same size as that used in the test. Add 1 ml. of freshly prepared pyrogallic–tannic acid solution to each and mix by inversion. Cover with a layer of melted paraffin and fill the remainder of the tubes with sealing wax. These standards are said to be permanent.

4. *Neutral Methemoglobin.* Dilute a little defibrinated blood (1:10) and add a few drops of freshly prepared 10 per cent solution of potassium ferricyanide. Shake this mixture and observe that the bright red color of the blood is displaced by a brownish red. Now dilute a little of this solution and examine it spectroscopically. Note the single, very dark absorption band lying to the left of D, and, if the dilution is sufficiently great, also observe the two rather faint bands lying between D and E in somewhat similar positions to those occupied by the absorption bands of oxyhemoglobin. Add a few drops of Stokes's reagent to the methemoglobin solution while it is in position before the spectroscope and note the immediate appearance of the oxyhemoglobin spectrum which is quickly followed by that of hemoglobin.

5. *Alkaline Methemoglobin.* Render a neutral solution of methemoglobin, such as that used in Exp. 4, above, slightly alkaline with a few drops of ammonia. Owing to the formation of alkaline methemoglobin the solution becomes redder in color and shows a spectrum different from that of the neutral solution. In this case we have a band on either side of D, the one nearer the red end of the spectrum being much the fainter. A third band, darker than either of those mentioned, lies between D and E somewhat nearer E.

DETECTION OF BLOOD STAINS ON CLOTH, ETC.

1. *Identification of Corpuscles.* If the stain under examination is on cloth, a portion should be extracted with a few drops of glycerol or physiological

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[31] Sayers and Yant: *Bureau of Mines Technical Paper 373* (1925). These authors describe a compact field apparatus. For gasometric determination of carbon monoxide in blood see Chapter 24.

[32] Made by dissolving 2 g. pyrogallic acid and 2 g. of tannic acid in 100 ml. of distilled water.

(0.9 per cent) sodium chloride solution. A drop of this solution should then be examined under the microscope to determine if corpuscles are present.

2. *Tests on Aqueous Extract.* A second portion of the stain should be extracted with a few drops of water and the following tests made upon the aqueous extract:

(a) HEMOCHROMOGEN. Make a small amount of the extract alkaline by potassium hydroxide or sodium hydroxide, and heat until a brownish-green color results. Cool and add a few drops of ammonium sulfide or Stokes's reagent (Appendix) and make a spectroscopic examination. Hankin has suggested a test based upon the formation of cyanhemochromogen and the microspectroscopic demonstration of the spectrum of this compound.

(b) HEMIN TEST. Make this test upon a small drop of aqueous extract according to the directions given on p. 483, or, better, make the test upon a little material scraped from the cloth and put directly on a slide.

(c) GUAIAC TEST. Make this test on the aqueous extract according to the directions given on p. 484. The guaiac solution may also be applied directly to the stain without previous extraction in the following manner: Moisten the stain with water, and after allowing it to stand several minutes, add an alcoholic solution of guaiac (strength about 1:60) and a little hydrogen peroxide or old turpentine. The customary blue color will be observed in the presence of blood.

(d) BENZIDINE REACTION. Make this test according to directions given on p. 484.

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23

Blood Analysis: Colorimetry and Photometry

The factors which influence the chemical composition of the blood in disease may be broadly classified as physical and metabolic. The former include those cases of retention due to alteration or destruction of permeable membranes in the excretory organs, such as the lungs, kidneys, and liver. The accumulation of nitrogenous waste products in certain forms of nephritis, and the hypercholesterolemia associated with obstruction of the biliary ducts by gallstones, are examples of retention brought about by such processes. In the so-called metabolic diseases alteration in the chemistry of the blood may be induced by increased or diminished formation or utilization of the various constituents. Thus the accumulation of glucose in the blood as a result of metabolic derangement is an outstanding feature of diabetes mellitus. In chronic nephritis with edema (nephrosis), the high cholesterol values are considered to be of metabolic origin. Without multiplying examples, suffice it to say that changes in the blood chemistry need not be anticipated unless some condition affecting formation, utilization, or elimination is suspected. It is noteworthy, however, that the line of demarcation between these factors cannot be drawn very sharply. For example, both metabolic and mechanical factors are probably at work in renal diseases. In this connection it is important to differentiate between cause and effect. For example, the evidence that gallstones are the result and not the cause of hypercholesterolemia is not complete.

The present status of clinical chemistry is largely the result of the development of methods for the satisfactory analysis of small amounts of blood. Prior to the advent of modern micromethods for blood analysis, quantitative knowledge of metabolic processes within the body was based primarily upon analysis of the diet and urine by the classical methods of analytical chemistry. This came about because the amounts of blood required for analysis by similar methods were so large as to preclude the routine use of blood analysis for clinical purposes, except for such isolated instances as the determination of hemoglobin. Shortly after the first decade of this century, the pioneer work of such outstanding biochemists as Folin, Benedict, Van Slyke, Myers, and their associates and pupils and many others, in developing methods for the analysis of small amounts of blood by volumetric, colorimetric, and gasometric methods, initiated the present phase of quantitative clinical chemistry in which blood analysis has become an indispensable adjunct to the study of the functions of the body in both health and disease.

The variety of blood constituents for which quantitative methods are available and whose analytical determination is now almost routine in many hospital laboratories and elsewhere is illustrated by the accompanying table, which lists the normal concentration range for many of the major constituents of human blood, together with the chief instances in which pathological variation may be encountered. The blood of other species of animals may vary with respect to certain constituents; for example, in the rat, nonprotein nitrogen is distinctly higher, while sugar, urea, and chlorides are slightly so.

For the quantitative analysis of blood and other biological material, in many cases a number of different methods are available for the determination of the same substance. This is because the aim of biochemical workers to evolve methods which are specific for the substances being determined has not always been successfully accomplished. Many methods have undergone (and are still undergoing) a process of evolution toward this goal. The early blood sugar methods, for example, were relatively nonspecific and gave values of 100 to 120 mg. of glucose per 100 ml. of blood in normal individuals. Later methods, by eliminating the effect of nonglucose reducing substances, give values lower by about 20 mg. per cent or more, and presumably nearer the true glucose content. Thus the "normal" value may depend to a great extent upon the method used, and knowledge of the method employed is essential in interpreting the significance of values obtained, particularly in the earlier literature. The obvious value of more specific methods cannot be underestimated, but for many purposes if an older and simpler but less specific method provides a guide to blood changes in pathological conditions, its usefulness continues.

Because of the large number of methods which have been described in the literature, in a number of cases for the same substance, the choice of methods described in this chapter is somewhat arbitrary. The authors have selected those methods which they believe to be most generally useful and satisfactory, the selection in practically all cases being based upon personal experience or contact with hospital and research laboratories. In many instances alternate methods are offered, to provide for differences of opinion concerning the relative status of methods, and for differences between laboratories in facilities available or the type of results required. Blood chemical methods differ considerably in the expense or complexity of the necessary apparatus, in the time required for preparation of reagents and the performance of the analysis, and in the degree of precision of the results obtained. These factors must be considered in the selection of a method. Certain methods, for example, are more adaptable than others to multiple determinations as required in large hospitals. For occasional clinical determinations where a relatively wider margin of error is admissible, some of the simpler but less accurate methods may be used. It is worthy of note, however, that the trend in modern clinical laboratories is toward more exact rather than less exact methods, since only in this way can the demands of modern medicine be met.

At the end of this chapter will be found a list of references to methods for the determination of blood constituents which are less commonly called for and which are not described in detail here or elsewhere in this

L1 - 9

COMPOSITION OF HUMAN BLOOD

1 - 3

Constituent	*Normal Range mg. per 100 ml.**	*Pathological Conditions in Which Increases (Unless Otherwise Noted) May Be Encountered*
Total solids, per cent	19–23	Anhydremia. Low in hydremic plethora and anemia.
Total proteins (serum), per cent	6.5–8.2	See above. Low in nephritis with edema (nephrosis).
Albumin (serum), per cent	4.6–6.7	Low in nephrosis.
Globulin (serum), per cent	1.2–2.3	Nephrosis, anaphylactic conditions, malignancy, infections, muscular activity.
Fibrinogen (plasma), per cent	0.3–0.6	Pneumonia, infections. Low in cirrhosis of liver, chloroform or phosphorus poisoning, typhoid fever.
Hemoglobin, per cent (Haden)	15.6	Polycythemia. Low in primary and secondary anemia, chlorosis.
Iron, as Fe	52	See Hemoglobin.
Copper	0.05–0.25	
Total nitrogen, per cent	3.0–3.7	Varies chiefly with proteins (albumin, globulin, hemoglobin).
Nonprotein N	25–35	Nephritis, eclampsia, etc. See Urea N.
Urea N	10–15	Chronic and acute nephritis, metallic poisoning, cardiac failure, intestinal or prostatic obstruction, some infectious diseases. Relatively low in nephrosis.
Uric acid	2.0–3.5	Nephritis, gout, arthritis, eclampsia.
Creatinine	1–2	Nephritis.
Creatine	3–7	Terminal nephritis.
Amino-acid N	5–8	Leukemia, acute yellow atrophy of the liver, severe nephritis.
Ammonia N	0.1–0.2	Terminal interstitial nephritis.
Undetermined N	4–18	Eclampsia.
Glucose	70–100	Diabetes, pregnancy, severe nephritis.
Total fatty acids	290–420	Diabetes, nephritis.
Cholesterol	150–190	Diabetes, nephritis, nephrosis, biliary obstruction, pregnancy. Low in pernicious anemia.
Lipide phosphorus	12–14	Diabetes, nephritis, pregnancy. In anemia, low in plasma, high in cells.
Total acetone bodies (as acetone)	0.8–5.0	Diabetes.
Acetone + acetoacetic acid (as acetone)	0.3–2.0	Diabetes.
β-Hydroxybutyric acid (as acetone)	0.5–3.0	Diabetes.
Bilirubin	0.1–0.25	Biliary obstruction, hemolytic anemias. Low in secondary anemia.
CO_2 capacity (plasma) vol. per cent	55–75†	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO_2 content (arterial blood) vol. per cent	45–55†	Respiratory diseases, tetany. Low in diabetes, nephritis.
CO_2 content (venous blood) vol. per cent	50–60†	Respiratory diseases, tetany. Low in diabetes, nephritis.
O_2 capacity vol. per cent	16–24†	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
O_2 content (arterial blood) vol. per cent	15–23†	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
O_2 content (venous blood) vol. per cent	10–18†	Polycythemia, anhydremia. Low in cardiac and respiratory diseases, anemia.
Ascorbic acid	0.8–2.4	Low in scurvy.
Lactic acid	5–20	Exercise, eclampsia.
Phenols (free)	1–2	Intestinal obstruction, pernicious anemia, nephritis.
Chloride as NaCl	450–500	Nephritis, cardiac conditions, prostatic obstruction, eclampsia, anemia. Low in diabetes, fever, and pneumonia.
milliequivalents per liter	77–86	
milliequivalents per liter (plasma)	98–106	
Sulfates, inorganic as S (serum)	0.9–1.1	Nephritis.
Phosphorus, inorganic as P(plasma)	3–4	Nephritis. Low in rickets. Normal values 1–2 mg. higher in children.
Calcium (serum)	9.0–11.5	Low in infantile tetany, severe nephritis, parathyroidectomy.
Magnesium (serum)	1–3	No changes noted in disease.
Sodium (serum)	300–330	Low in cases of alkali deficit.
milliequivalents per liter	130–144	
Potassium (serum)	16–22	Pneumonia, acute infections, occasionally uremia.
milliequivalents per liter	4.1–5.6	
Iodine (total), micrograms per 100 ml.	8–15	Hyperthyroidism. Low in cretinism.
Protein-bound (serum), micrograms per 100 ml.	4–8	Hyperthyroidism and pregnancy. Sometimes low in hypoproteinemia.

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* Figures express concentration in mg. per 100 ml. of whole blood unless otherwise indicated in the first column.

† Figures represent weighted averages of the observations of several investigators.

book. There is also a selected list of books and other treatises to which the reader is referred for a more comprehensive discussion than is possible in these pages of certain of the subjects considered here, particularly with reference to historical aspects and the clinical significance of laboratory data.

COLORIMETRY AND PHOTOMETRY

Introduction. Many methods for the quantitative analysis of blood, tissues, urine, and other biological material are based upon the production of colored solutions in such a way that the intensity or depth of color so obtained may be used as a measure of the concentration of substance being determined. Such use of color as an index of concentration has long been known to analytical chemists as the science of colorimetric analysis, or colorimetry, and the instrument used for color evaluation is called a colorimeter. These terms, while admittedly not precise, have the sanction of established usage, particularly when they refer to the very common type of color measurement in which the colored solution representing the substance in unknown concentration is brought by one means or another to exact color match with a suitable standard color. Until relatively recently this was the only practical method for colorimetric analysis, because the human eye is much more capable of establishing the presence or absence of exact color equivalence than it is of defining quantitative differences in color intensity in precise terms.

It is clear, however, from a knowledge of the physical nature of color, that color intensity may be established in terms of the degree of light absorption at specific regions or wavelengths in the visible spectrum. To take a simple example, a solution has a blue color because generally speaking it absorbs a lesser proportion of the blue components of the mixed white light passing through it than of any of the other colored components. Thus white light entering the solution will emerge in diminished intensity and have a preponderance of blue wavelengths. The darker the solution, i.e., the more intense the color, the greater must be the degree of light absorption at certain wavelengths, so that such light absorption may be used as a direct measure of color intensity. Analytical procedures based upon the direct measurement of color intensity in terms of light absorption at specific wavelengths are known as photometric procedures, and the instrument used is called a photometer. This usage is by no means universal, however, and there are many who continue to use the term "colorimetric" for methods based upon the production of colored solutions, regardless of the means by which the color intensity is established. To avoid confusion, it appears desirable to define a *colorimetric procedure* as one in which the colored solution representing the substance in unknown concentration is brought to exact color match with a standard color representing the substance in known concentration, while a *photometric procedure* is one based upon the direct measurement of color intensity in terms of the light-absorbing power of the solution at a specific region of the spectrum. Unlike colorimetric procedures, which are limited to the visible portion of the spectrum, the general principles of photometric procedures are as applicable to the absorption of radiant energy in the ultraviolet and infrared portions of the spectrum as they are to absorption in the visible region, and increasing analytical use is being made of this fact. The use of *turbidity* and *fluorescence* for analytical purposes is discussed on pp. 533 to 537.

COLORIMETRY

As defined above, colorimetry is based upon the matching of a colored solution representing an unknown concentration of the substance undergoing analysis with a standard color representing the substance in known concentration. The substance must therefore be either colored by itself or capable of undergoing reactions leading to the production of a color. Furthermore, the color intensity must be dependent upon the concentration, otherwise the color reaction is valueless for colorimetric purposes. A colorimetric procedure therefore involves three operations: (1) The preparation of the colored solution to represent the unknown, (2) the obtaining of a suitable standard color, and (3) color matching.

If the substance being determined is itself colored—as, for example, hemoglobin, carotene, certain inorganic ions, dyes, etc.—the preparation of the colored solution for analysis is usually relatively simple, and may involve merely appropriate dilution or concentration of the sample to produce a color of intensity suitable for comparison against a standard. Even under these conditions, however, it is often better to separate the colored compound from possible interfering colored or noncolored material prior to estimation. If the substance must undergo a series of reactions leading ultimately to the production of a color, it is of the utmost importance to recognize that the final color intensity may be influenced to a considerable extent not only by the concentration of unknown substance but also by the intermediate steps leading up to and including the development of the color. Such factors as the time of heating and cooling, order and rate of addition of reagents, whether the reagents are new or old, the time of standing and temperature of the solution during color development, the presence of nonchromogenic material such as neutral salts, and even the volume of solution in which the color reaction occurs, are all known to influence the final color intensity for a given amount of material in many if not all colorimetric procedures. For accurate and reproducible results, therefore, it is essential that all steps in a colorimetric procedure be carried out under conditions as carefully controlled as possible. In many cases the authors of colorimetric procedures have carefully specified the conditions for precise analysis, and these conditions should be followed without deviation.

The obtaining of a suitable standard color is obviously a most important phase of a colorimetric procedure. It may be stated without qualification that the most satisfactory standard color, and the one which should always be used for accurate results, is that obtained by treating a known concentration of the substance being determined by exactly the same procedure that is used for the unknown, at the same time, and under as nearly identical conditions as possible. Thus the final colors in standard and unknown will be due to the same substance, differing if at all only in intensity, and the many nonspecific factors already mentioned which may influence color intensity will presumably affect the standard and unknown to exactly the same extent and will not influence analytical results based upon their comparison. All colorimetric procedures must be originally based upon the use of this type of primary standard, even though secondary standards may be used later, as discussed below. This results because there is no way to predict the relationship between color intensity and concentration except in terms of the color yielded by a known concentration of the substance.

It is assumed in the use of a standard color that if the standard and unknown exactly match in color intensity they represent equal concentrations of the substance being determined. In actual practice this may or may not be true. The standard usually contains the substance being determined in relatively pure solution; in the unknown, extraneous factors may be present which modify color intensity. Substances other than the one being determined may enter into the color reaction, and results will therefore be too high; the analytical problem under these conditions is to devise either a more specific color reaction or to find methods for eliminating nonspecific interfering substances, and much of the trend in colorimetric analysis has developed along these lines. Another type of interference which is less frequently recognized is the influence of nonchromogenic material present on the intensity of color produced by a given amount of chromogenic substance. This may be tested for by adding to the unknown a given amount of the pure substance, and measuring the resulting increment in color intensity. If this increment is greater or less than that known to represent the added amount of substance in pure solution, and there is no possibility of loss or destruction, then factors are present in the unknown which modify color intensity per unit concentration, and suitable correction must be made. This method of using an "internal standard," as it is sometimes called, does not prove that the total color yielded by the unknown is due to the substance being determined, but it does establish whether or not this substance is capable of giving a complete color reaction under the conditions of the analysis.

In some colorimetric procedures standard solutions are required containing substances which are expensive or difficult to obtain in the pure state (e.g., bilirubin, and at one time, creatinine), or which deteriorate rapidly on standing or may require unavailable technical skill and apparatus for standardization (e.g., hemoglobin). To provide for the routine use of colorimetric procedures based upon such standards, various "artificial" standards have been devised. In most instances these standards consist of stable colored solutions of dyes or inorganic salts, or of colored glass or gelatin. The color is selected by the investigator or manufacturer to correspond as closely as possible to that representing a known amount of the substance being determined. Examples of the use of artificial standards will be found in the Newcomer method for the determination of hemoglobin (p. 616), in the Benedict picrate method for the determination of urine sugar (p. 923), and elsewhere. In using such standards, the color in the solution being analyzed is developed by the usual procedure and then compared against the artificial standard or standards representing known amounts of material.

There are many difficulties in the way of obtaining accurate results with artificial standards. Aside from the technical problem of an exact color match—and few individuals agree with one another on this point—it has already been pointed out that color intensity depends not only upon concentration but also upon the technique of the analytical procedure. Therefore the intensity of color corresponding to a given amount of material being determined may vary from laboratory to laboratory. Furthermore, few colorimetric procedures result in the production of a color which does not show either an increase or a decrease in intensity on standing. Comparison against a simultaneously prepared standard which undergoes equivalent changes in color intensity will eliminate errors due to such changes; comparison against a stable standard will clearly give results which may depend largely upon the time of standing after color development, and careful control of this factor may therefore be necessary. With few exceptions, artificial standards are satisfactory only where approximate results are sufficient; they should never be used simply to relieve the analyst of the responsibility for preparing and maintaining an exact standard solution. If artificial standards are used, they should always be checked in one's own laboratory and with one's own reagents, to eliminate errors from inexact calibration, and this checking should be repeated at frequent intervals or when new reagents are prepared.

Colorimeters. Colorimeters are instruments used to facilitate the exact matching of two colored solutions. This matching may be done in a variety of ways, summarized as follows:

1. BY COMPARISON AGAINST A SERIES OF STANDARDS. The unknown colored solution is compared by inspection with a series of color standards representing the substance being determined in known and varying concentration. The concentration of the unknown is given by the concentration of the standard which it exactly matches. The colorimeter is simply a device for holding the standards and the unknown and for providing uniform conditions of illumination to facilitate exact color match. The method is simple and requires relatively inexpensive apparatus. Chief drawbacks include the labor of preparing and maintaining the standards, the possibility of error due to deterioration of standards already prepared, and the fact that the range and precision of the method are limited by the number of standards available. The method therefore finds greatest application where it is known that the scope of the analytical problem is limited to results within a certain range of concentration, and highest accuracy is not required. Examples of the use of this principle are found in the colorimetric determination of hydrogen-ion concentration (Chapter 1), in the Benedict picrate method for urine sugar (p. 923), and in an increasing number of "pocket," "bedside," and "field" analysis outfits available commercially for specific analytical purposes (see Fig. 121). In many instances these outfits are fitted with artificial permanent standards, usually of colored glass or gelatin, to eliminate the necessity of preparing a series of standards. The use of such artificial standards has already been discussed.

2. BY DUPLICATION OF COLOR. In this method a relatively concentrated standard solution is measured into a "blank" containing the same

reagents as used in the sample until the color matches that of the sample, after the volume of the standard has been brought up to the volume of the sample by the addition of distilled water and thoroughly mixed. The volume of standard solution required to prepare the duplicate is a measure of the amount of test substance in the sample. Some authors call this method *colorimetric titration.*

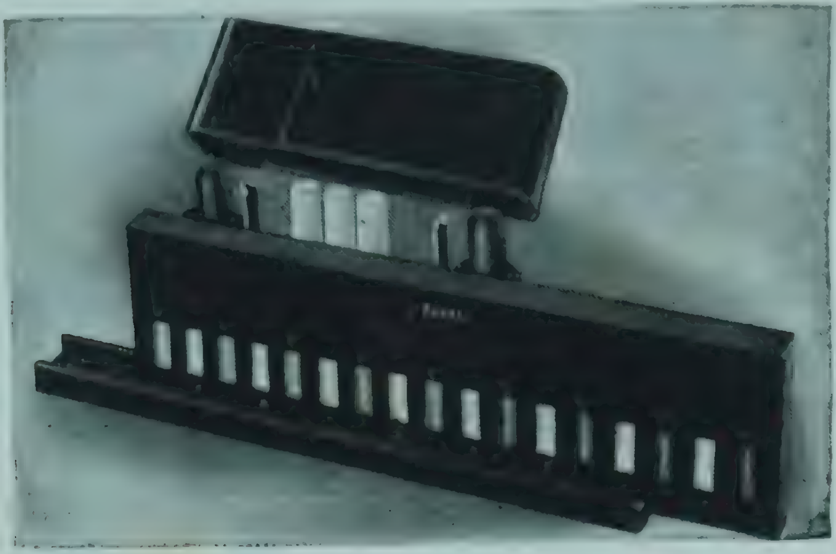

FIG. 121. SLIDE COMPARATOR, FOR COLORIMETRIC COMPARISON AGAINST A SERIES OF STANDARDS.

Courtesy, W. A. Taylor Co.

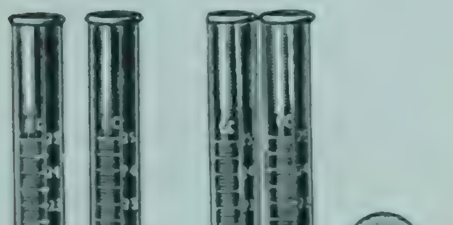

FIG. 122. MYERS' TEST-TUBE COLORIMETER.

3. BY DILUTION TO COLOR MATCH. Either the unknown or the standard is diluted with water or other solvent until the two colors exactly match in intensity when compared under similar conditions of illumination and depth of solution through which the light passes. Thus if the unknown must be diluted to twice its original volume to match a given standard, its original concentration is assumed to have been twice that of the standard, and in general if two colored solutions differing only in concentration are brought to color match by diluting one of them, the original concentration of the diluted solution is equal to the concentration of the undiluted solution multiplied by the ratio of final to initial volumes for the diluted solution. Only the simplest of apparatus is required for this procedure; the colorimeter may consist of two graduated test tubes (Fig. 122) or cylinders of equal bore. In the Sahli "Hemoglobinometer" (see Fig. 161, p. 612), which exemplifies this type of color comparison, the blood sample is diluted in a graduated tube to match a permanent standard representing a known concentration of hemoglobin; from the dilution required the hemoglobin content of the sample is directly determinable. Dilution colorimetry is somewhat tedious, being comparable to a volumetric titration to an arbitrary end point, but results with an error not exceeding 5 per cent may

be obtained with practice. A major disadvantage of the dilution procedure is that many colored solutions are affected by dilution out of direct proportion to the change in total volume, owing to the influence of such factors as neutral salt concentration, acidity, or even the relative amount of solvent itself on the color intensity. Thus a blue solution of copper sulfate on being diluted to twice its original volume will have its color intensity affected not only by the change in volume but also by the change in copper-ion activity ("degree of dissociation") brought about by the dilution. It cannot be assumed that the dilution procedure is accurate, therefore, unless it has been shown to be so experimentally.

4. BY VARYING THE DEPTH OF SOLUTION THROUGH WHICH THE LIGHT PASSES. This procedure is the basis of the common laboratory visual colorimeter, and is called the *balancing method* of color comparison. It is based upon what is commonly known as Beer's law, which for present purposes may be stated as follows:[1] the intensity or density of color in a solution is determined for a particular substance solely by the number of colored particles (molecules or ions) in the light path. For example, a 1 per cent solution of a dye viewed through a solution depth of 20 mm. should have the same color as a 2 per cent solution viewed through 10 mm. of solution, because there are the same number of colored molecules or ions in the light path in each instance. In other words, color density is directly proportional to the concentration of colored substance and the depth of solution through which the light passes. Stated mathematically, for a particular colored substance,

$$D = k \times c \times l$$

where D is the color intensity or density, k is the proportionality constant characteristic of the substance, c is the concentration of colored substance, and l is the length or depth of solution traversed by the light beam.

For two solutions of the same substance at different concentrations c_1 and c_2, and depths of solution l_1 and l_2,

$$D_1 = k \times c_1 \times l_1$$

and

$$D_2 = k \times c_2 \times l_2$$

If l_1 and l_2 are varied so that the two solutions have the same color intensity (i.e., $D_1 = D_2$), which is what is done in colorimeters based upon this principle, then

$$k \times c_1 \times l_1 = k \times c_2 \times l_2$$

or, by transposing and canceling out k,

$$\frac{c_1}{c_2} = \frac{l_2}{l_1}$$

Thus two different concentrations of the same colored substance are related inversely to the depths of solution required for color match. If one of these solutions is a standard of known concentration, C_S, adjusted to color match with the solution of unknown concentration, C_x, by varying the depth of solution (readings R_s and R_x respectively on the colorimeter), the equation becomes:

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[1] See pp. 515 to 522 for a more detailed discussion of Beer's law.

$$\frac{C_x}{C_s} = \frac{R_s}{R_x}$$

or

$$C_x = \frac{R_s}{R_x} \times C_s$$

i.e., the *concentration of the unknown is given by the readings of standard and unknown and the concentration of the standard.*

In using this equation, it must not be overlooked that the term *concentration* means *amount per unit volume*, hence a more exact form of the equation is as follows:

$$\frac{X}{V_x} = \frac{R_s}{R_x} \times \frac{S}{V_s}$$

or

$$X = \frac{R_s}{R_x} \times S \times \frac{V_x}{V_s}$$

where X and S are the actual amounts of substance present in volumes V_x and V_s of unknown and standard colored solutions respectively, and R_x and R_s are the colorimeter readings as before. This form of the equation is the fundamental one upon which are based all colorimetric procedures of the type described here. In most procedures the final volumes of unknown and standard are the same, and the volume factor V_x/V_s cancels out; results are then obtained primarily in terms of the actual amounts of substance present, and as a matter of fact the directions for most colorimetric procedures prescribe the taking of a definite amount rather than concentration of standard. In certain colorimetric procedures (e.g., the determination of creatinine in blood, p. 555) the volumes of standard and unknown differ, and in such cases these volumes must be substituted in the above equation.

The value of X as obtained by the above calculation represents the amount of material in the portion of sample actually taken for color development. To express results in terms of amount per 100 ml. of blood, for example, X must be multiplied by $100/v$, where v is the volume of blood in ml. which contained the material on which the color was developed. Thus if color development in a blood sugar determination is carried out on a 2-ml. portion of filtrate from the blood diluted tenfold in the preparation of the protein-free filtrate used for the actual analysis, v in this case equals 0.2, since the 2 ml. of filtrate represent 0.2 ml. of original blood.

In applying the above equations to colorimetric calculations, certain limitations must be noted. For mechanical, optical, and analytical reasons the inverse relationship between concentration and depth of solution at color match is in general not applicable over the entire length of the colorimeter scale, which is usually about 40 to 50 mm. long and graduated in millimeters. The standard is ordinarily selected to be of such strength as to give a good intensity for color comparison at a depth of about 15 or 20 mm. It is a general rule that readings of an unknown which are less than half or more than double the reading of the standard are outside the range of application of Beer's law. The amount of sample taken for analysis is selected if possible so that the expected reading will come within this range relative to the standard reading; readings outside this range are regarded as approximations only, to be used as a guide for repeating the analysis on a more satisfactory aliquot of sample. In general, it is better to change the amount of sample analyzed so as to come within the range of the standard than to alter the standard, because the standard is usually so selected as to provide the most satisfactory intensity for color match; lighter or darker standards may give less accurate results. If the amount of sample available is limited, however, and it is known that unusually high or low values may be encountered, the analysis may be saved by routinely providing several standards at different concentrations, the unknown being compared against the standard which it most closely matches on inspection.

In some colorimetric procedures the range of inverse proportionality between scale reading and concentration is much less than that represented by the "half or double" rule. The authors of such procedures usually specify the reliable range of readings. If the deviation from Beer's law is systematic, it is sometimes possible to establish a table of corrected values, showing the relation between the observed readings and the amount present, thus extending the range of permissible readings. From what has been said concerning the various factors which influence color intensity, however, it is clear that such a table is highly empirical and usually reflects the conditions prevailing in one laboratory only. It should be checked at intervals for accurate results; this checking is particularly important if a table developed elsewhere is to be used.

The use of light filters or of a monochromatic light source is ordinarily unnecessary in colorimetric comparisons, and mixed white light is commonly used, since if standard and unknown contain the same colored substance, at color match light transmission must be the same at all wavelengths. If extraneous colored material is present (i.e., if the reagents themselves are colored), or if unknown and standard differ in *hue* as well as in *intensity*, properly selected light filters may considerably improve the precision of readings or the range of applicability of Beer's law. When artificial standards are used, uniform and reproducible illumination is particularly important, because such a standard may, for example, represent one concentration by daylight and a different concentration by artificial light.

Colorimeters constructed to utilize the relationship between depth of solution and concentration expressed in Beer's law usually consist of (*a*) a source of light (mirror or built-in electric lamp); (*b*) a pair of adjustable cups and plungers, for varying the depth of solution through which the light passes; (*c*) an optical arrangement for looking down through the plungers and for bringing into juxtaposition the two fields of light from the solutions being compared, to facilitate exact color match. Many

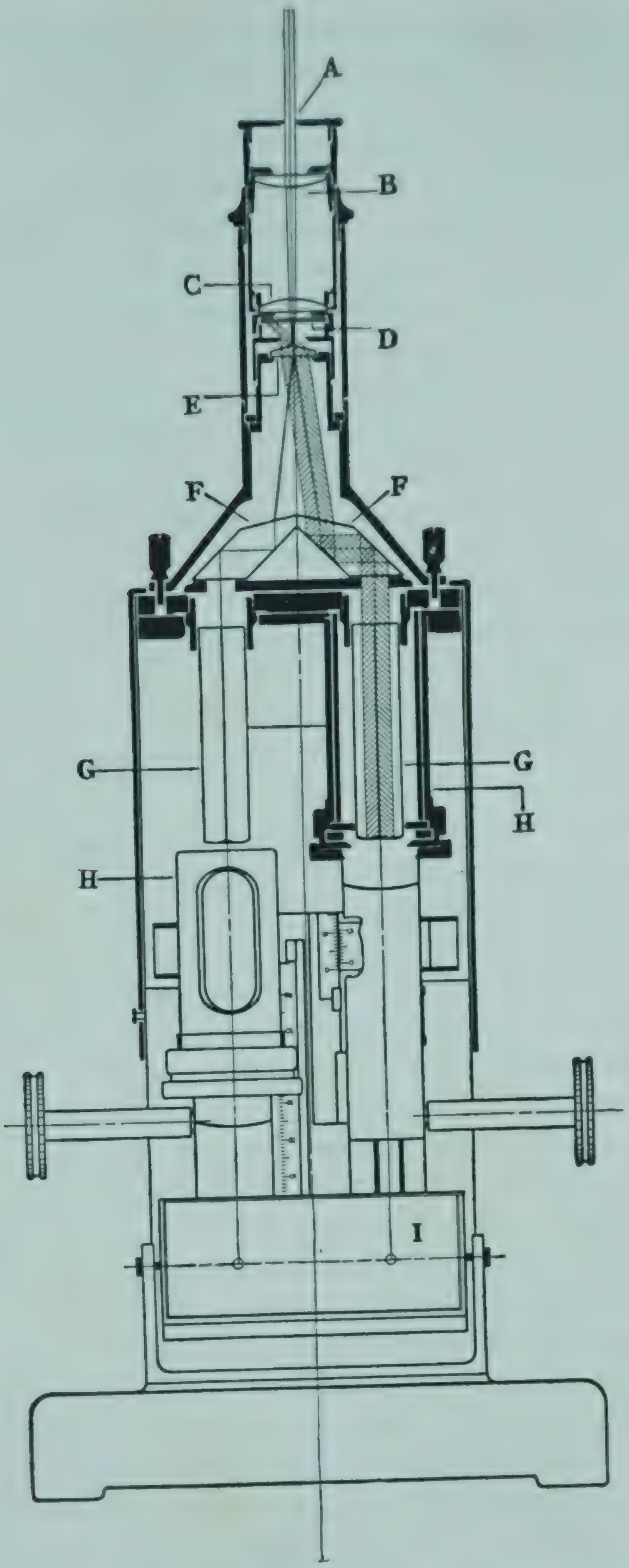

FIG. 123. DIAGRAM SHOWING CONSTRUCTION OF DUBOSCQ COLORIMETER (BAUSCH AND LOMB).

A, Eye point; *B*, eye lens; *C*, collective; *D*, cover glass; *E*, bi-prism; *F*, rhomboid prism; *G*, plungers; *H*, cups; *I*, mirror.

different commercial models are available.[2] The constructional details of one type are shown in Fig. 123, and other types are illustrated in Figs. 124 and 125. In the original colorimeter of this type, designed by Duboscq, the cups were fixed and the plungers were raised or lowered to vary depth of solution; in modern Duboscq-type colorimeters, the plungers are fixed and the cups are adjustable. The cups usually hold about 5 ml. of solution; micro-cups and plungers may be used for smaller volumes. Cups with flared tops are preferred over the straight-side type. Some types of cups leak when in contact with certain nonaqueous solvents such as chloroform, and are therefore unsuitable for such solvents unless sealed with a resistant cement. For illumination, the most satisfactory sources are north sky light or light from an electric lamp equipped with a "daylite" filter. In selecting a colorimeter, the choice should be determined largely by size and definition of the optical field and the evenness of its illumination, excellence of mechanical and optical construction, and ease of operation and reading.

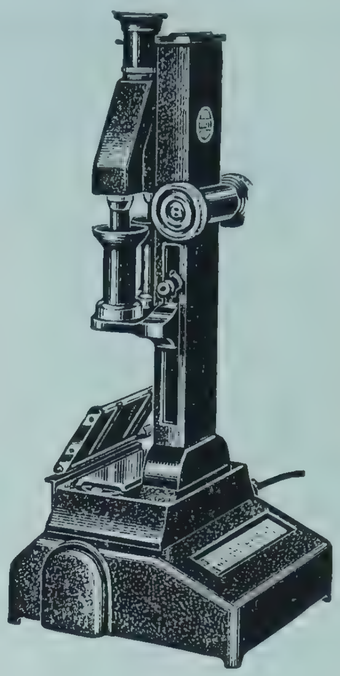

FIG. 124. KLETT BIOCOLORIMETER, WITH BUILT-IN LAMP IN BASE.

Courtesy, Klett Manufacturing Co.

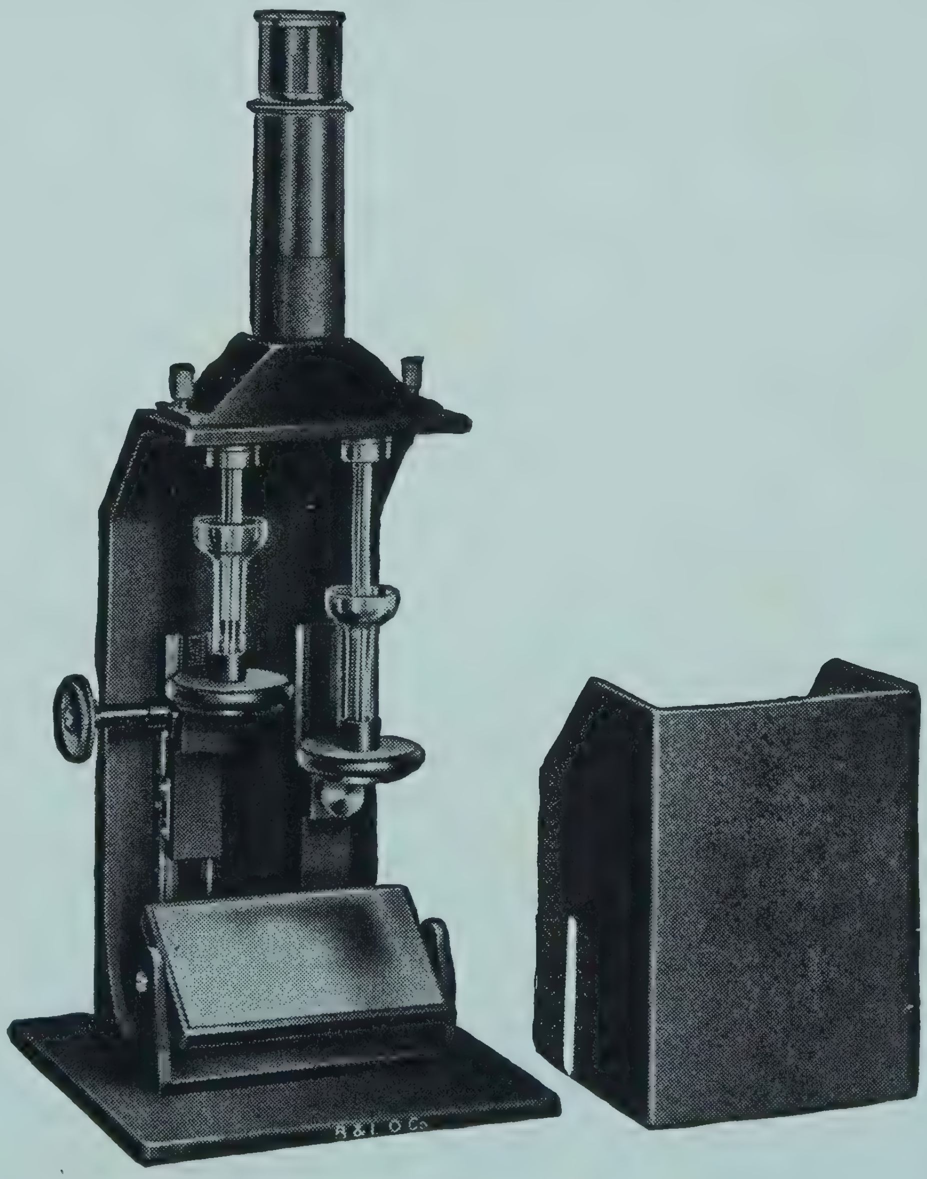

FIG. 125. MICROCOLORIMETER (BAUSCH AND LOMB).

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[2] The instruments manufactured by Klett Manufacturing Co., New York, Bausch and Lomb Optical Co., Rochester, N. Y., and American Optical Co., Buffalo, N. Y., are especially satisfactory in the authors' experience.

The general principles involved in the use of a Duboscq-type colorimeter are illustrated by the following experiments.

EXPERIMENTS ON COLORIMETRY

1. *Preparation of Colorimeter for Use:* (a) LIGHT SOURCE. Place the colorimeter on a firm table with the mirror facing the light source. A north window or "daylite" lamp is best. Some colorimeters have a built-in electric lamp in the base; this is equally satisfactory. Look through the eyepiece of the colorimeter and note the appearance of the field, focusing if necessary to bring it into sharp definition. Adjust the mirror (or the lamp in the base) and note that the evenness and intensity of illumination can be varied at will by this adjustment. Adjust until the two halves of the field are approximately evenly illuminated, at maximum intensity. This is a preliminary adjustment only; final adjustment in an analysis must always be made with the standard, as described in Exp. 2.

(b) CHECKING THE ZERO. Place each cup, clean and dry,[3] on its rack beneath a plunger, and carefully rack up the cup until it comes in contact with the bottom of the plunger. Read the colorimeter scales; each should read **0.0**. If they do not, locate the scale adjustment or its equivalent and adjust so that both scales read **0.0** when cup and plunger are in contact. Check by lowering the cups slightly, then raising to contact, and again reading. Now lower the cups and interchange them on the plungers. Again bring to contact with the plungers and read. Are the scales still in adjustment? It is common practice to mark one cup, as by wrapping a rubber band around it, so that accidental exchange will not occur.

2. *Use of Colorimeter for Comparing Two Solutions.* Obtain two colored solutions, a standard and an unknown, containing the same substance in slightly different concentrations. Half fill each cup with a portion of the standard, place the cups on the racks, and rack up carefully to contact with each plunger. This serves to displace air bubbles which may be trapped under the plunger. Get into the habit of routinely checking the zero at this point at the same time. Lower the cups until both scales read exactly 15.0. Look through the eyepiece. Adjust the light source carefully until the two halves of the field are as evenly illuminated as possible. Theoretically, the reading on each side is now 15.0; actually, there is as much error in this adjustment as in the subsequent reading of an unknown; therefore for precise results the solution on one side should now be read against the other side ("matching the standard against itself"). Leaving the left-hand cup set, lower the right-hand cup slightly to throw the two halves of the field out of balance, then raise the cup slowly while looking through the eyepiece, until the field appears exactly even. Note the right-hand scale reading. Repeat this process three or four more times, and average the readings. *This is the actual reading of the standard that is used in the calculations.*

When the reading of the standard in the right-hand cup has been established, remove this cup, discard its contents, and place a portion of the unknown in the cup. Holding the cup in the hand, run it up and down briefly on the right-hand plunger, thus rinsing cup and plunger with the unknown. Discard the cup contents and repeat the rinsing with a fresh portion. Finally half fill the cup with fresh unknown and replace it on the cup rack. Run it up carefully to contact with the plunger, to displace air bubbles, then lower it

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[3] Use a fresh piece of soft, lintless paper (lens paper or "Kleenex") to wipe the bottom of each cup dry. Do not use a towel or handkerchief for this purpose.

until inspection through the eyepiece indicates color match. Make the reading, and repeat three or four times as described for the standard, averaging the readings. ***The average result is the reading of the unknown.***

From the readings of standard and unknown, and the known concentration of the standard, the concentration of material in the unknown is calculated as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \begin{matrix}\text{Concentration}\\ \text{of Standard}\end{matrix} = \begin{matrix}\text{Concentration}\\ \text{of Unknown}\end{matrix}$$

Note that in this calculation the readings of standard and unknown are both made on the same scale, i.e., the right-hand scale. The standard in the left-hand cup is set at 15.0 (or 20.0, or whatever value is specified) merely to establish a satisfactory color intensity for comparison purposes, and its reading does not enter into the calculations. As a matter of fact, some types of colorimeter do not have more than one scale. It is true that various other methods of reading a colorimeter have been proposed, but these are all subject to more error than the procedure described here.

In making colorimeter readings, it is good practice to rest the eyes frequently by looking up from the colorimeter, because the eyes tire easily and color discrimination becomes less acute. Make four or five independent readings and average them to minimize error; if one reading in the series is obviously quite different from the others it may be discarded in averaging. Best results are obtained if alternate readings are approached from above and below, and the hand removed from the instrument between readings, thus minimizing the tendency to return mechanically to the same reading.

3. Influence of "Personal Error" on Colorimetry. **Repeat the above experiment, but after you have read the standard against itself, have another individual make independent readings, and compare results. How closely do you agree? Now read the unknown against the standard, and calculate the concentration of the unknown from your readings. Have the second person likewise read the unknown and calculate results from his readings. Is any difference in opinion concerning the readings reflected in the final results? In general, it is necessary for each analyst to make his own readings of both standard and unknown; in this way, differences of opinion as to precise color match do not influence results.**

4. Beer's Law. **Obtain a series of aqueous methylene blue solutions containing 0.004, 0.005, 0.010, 0.020, and 0.030 g. per liter respectively of the dye. Select the 0.010 g. per liter solution as a standard, and set at 15 mm. Match the standard against itself, making the readings as described in Exp. 2, and then read each of the other solutions against this standard. On a sheet of cross-section paper, plot the colorimeter readings on the** ***y*****-axis against the concentration on the** ***x*****-axis. Draw a smooth curve between the points. Now calculate by the use of Beer's law what the various readings should be for the determined reading of the standard. Plot these theoretical readings on the same graph, and connect the points by a smooth curve. Do the two curves overlap completely or do they diverge at any points? To what extent does this dye follow Beer's law under these conditions? How could you obtain accurate results in a colorimetric procedure which is known not to obey Beer's law?**

Another way of plotting results is to plot the theoretical reading for a given concentration against the actual reading. A straight line indicates adherence to Beer's law. It is the general practice to obtain results by calculation based on Beer's law only if the unknown reading is not more than double or less than half the standard reading. Unknowns falling outside this range are repeated with more or less of the sample for analysis.

5. *Influence of Analytical Procedure on Color Intensity.* Into each of two small flasks place 20 ml. of standard ammonium sulfate solution containing 0.2 mg. of nitrogen (see p. 549). To one flask add 2 ml. of Nessler's reagent,[4] drop by drop, from a buret. (Because of its poisonous nature,[5] never use a pipet with Nessler's reagent.) Set aside, and to the second flask add 2 ml. of the Nessler reagent, as rapidly as possible. Mix by lateral shaking and set aside. After 10 minutes' standing, read one of the two solutions against itself in the colorimeter, and then read the second solution against the first. Do the readings indicate that the two solutions have the same concentration of ammonia? What must be done to ensure uniform results?

PHOTOMETRY

Photometry, in the sense in which the term is understood in analytical chemistry, consists in the measurement of the light-transmitting power of a solution in order to determine the concentration of light-absorbing material present. For purposes of simplicity the term *light* is used in place of the more inclusive term *radiant energy*. It must be understood that the principles upon which photometry is based are as applicable to the transmission of energy in the ultraviolet or infrared portions of the spectrum as they are to transmission in the more commonly employed visible, or colored, spectrum (see Fig. 287, p. 1249, for a chart showing the spectral distribution of radiant energy).

The ability of a solution to transmit light is known as the transmittance, T, of the solution. Strictly speaking, *transmittance* is defined as the ratio of the intensity, I_2, of the light emerging from the cell containing the solution to the entering or incident light intensity, I_1, or

$$T = \frac{I_2}{I_1}$$

For photometric purposes, however, it is neither practical nor necessary to measure the incident light intensity and to define transmittance in this way. In a photometric procedure there is always a certain proportion of nonspecific light loss during passage through the solution due to reflection from glass surfaces, scattering, and absorption by the solvent, reagents, and even possibly by contaminating traces of the substance which is being determined. Any of these will obviously influence a single measurement but should be constant from one measurement to another, and may therefore be balanced out by defining transmittance in *relative terms*. Thus if we let T_{soln} be the (over-all) transmittance of a solution containing a light-absorbing material (the solute) and let T_{solv} be the (over-all) transmittance of a reference solution (the solvent), usually the colorless solvent or reagent blank, both solutions being examined under equivalent conditions of wavelength, incident light intensity, and depth of solution, then the ratio of the transmittance of the solution to that of the solvent (or reagent blank) is equal to T_s, the *transmittancy*[5a] of the sample, i.e.,

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[4] See Appendix.

[5] See Chapter 31 for a discussion of the chemistry of Nessler's reagent and the reaction with ammonia.

$$T_s = \frac{T_{soln}}{T_{solv}} = \text{Transmittancy}$$

In this way neither the intensity of the incident light nor the nonspecific light loss need be determined, and furthermore contaminating traces of the substance being determined, or reagents which are themselves colored, do not interfere in an analysis. The change in transmittance due to the presence of the substance is determined solely by the increase in light absorption above a level which is *arbitrarily taken as zero*. It is this possibility of ruling out error from blank light absorption which represents an outstanding advantage of photometric analysis over colorimetric analysis with the Duboscq-type colorimeter, where this cannot be carried out without the use of correction factors.

Transmittancy, T_s, is thus a relative measurement and is always less than 1.00 if light-absorbing material is present. It may be expressed numerically either as a decimal fraction or in terms of per cent, e.g., a transmittancy of 0.65 or 65 per cent. A more satisfactory way of expressing the transmittancy of a solution is in terms of its negative logarithm,[6] $-\log T_s$, known as the *absorbancy*, A_s, also designated as the optical density, D, or the extinction, E, of the solution.

$$A_s = -\log T_s = \log \frac{1}{T_s} = \text{Absorbancy}$$

The utility of this basis for defining light absorption, particularly for photometric purposes, will be presented subsequently.

Determination of Transmittancy. The light transmittancy of a solution is determined by the use of an instrument known as a photometer. Many varieties and designs of photometers have been described and are available commercially; regardless of design, the principle upon which all analytical photometers operate is fundamentally the same, and may be described as follows: Light of suitable wavelength is allowed to pass through a reference solution, usually the colorless solvent or reagent blank, held in a container of fixed dimensions known as a cuvette. The intensity of light emerging from the reference solution is established at an

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[5a] It is felt by some that a distinction between *transmittance* and *transmittancy*, as defined here, is relatively unimportant. Until standardized nomenclature in this field becomes more universally adopted, one may find some authors or publications using one term and some the other when referring to photometric measurements.

[6] Some investigators, particularly in European laboratories, use the natural logarithm rather than the common logarithm, i.e., $-\ln T$ rather than $-\log T$. The choice is immaterial for photometric purposes but may lead to confusion in applying data obtained elsewhere. The relation between these two methods of expression is as follows:

$$-\log T = 0.4343 \times (-\ln T)$$

arbitrary value by any of the various methods described below, this value usually corresponding to a reading on the photometer scale of 0 optical density or 100 per cent transmittancy. The reference solution is then replaced by the solution whose transmittancy is to be determined, held in the same or a similar cuvette, and the emergent light intensity measured relative to that established for the reference solution; this relation gives the transmittancy of the solution under examination.

The intensity of light emerging from a solution may be established by either *visual*, *photographic*, or *photoelectric* (or equivalent) means; of these, the last is most common, most accurate, and has largely displaced the others. In a visual photometer, the emergent light beam is compared in intensity with a parallel reference beam of similar properties which is of arbitrary and adjustable intensity. The adjustment required to bring the reference beam to the same intensity as that emerging from the solution under examination is the measure of the emergent light intensity. Measurements are therefore influenced by the acuity of visual color-intensity discrimination, as in visual colorimetry. In the photographic plate method, the intensity of action of the emergent light on a photographic plate is compared with the action of light of relatively known and adjustable intensity under similar conditions. This procedure is tedious and has been used in the past largely for measurements in the ultraviolet and infrared portions of the spectrum, where the eye is insensitive. Much of the earlier data in the literature concerning light absorption in these regions of the spectrum is based on this method, but it has been superseded almost entirely by the use of light-sensitive devices such as the photoelectric cell or its equivalent.

Light intensity is determined photoelectrically by using photoelectric cells or similar light-sensitive devices which produce an electric current in proportion to the intensity of light striking their active surfaces. Two types in common use are (1) the photovoltaic cell ("plate type," "rectifier," or "barrier layer" cell) and (2) the photoemissive tube. A photovoltaic cell consists essentially of a metal plate coated with light-sensitive material (selenium, cuprous oxide) which is in turn coated with a thin transparent film of a metal such as gold or copper. Light passing through the transparent film sets up a flow of electrons in one direction ("rectified") which establishes a potential difference between the two poles of the cell, and causes a current to flow if the cell is in a suitable electric circuit. Because of the electrical characteristics of photovoltaic cells, the current is not suitable for amplification, but for ordinary light intensities it is sufficiently large to be registered on a microammeter or low sensitivity galvanometer, and many types of photometers employ such cells. Photoemissive tubes or phototubes are either evacuated or gas-filled tubes similar in appearance to radio tubes and containing a plate coated with some substance which emits electrons when light strikes it, the electrons traveling to a suitable anode under proper conditions. The intensity of incident light thus determines the flow of electrons through the tube, and hence the current in an external circuit. This current is very small but may be readily amplified, and photometers employing such tubes usually have an amplifier circuit. An advantage of phototubes is that they may be obtained with wide ultraviolet and infrared sensitivity, which is not the case with the photovoltaic cell.

With photosensitive devices, change in current output is used as a measure of change in light intensity. If the current output is adjusted to an arbitrary value with the reference or blank solution in place in the photometer, then the transmittancy of an unknown solution is given by the ratio of current output for this solution as compared to that for the reference solution. It is assumed that the current output is strictly proportional to the light intensity; with well-designed photometers this is usually sufficiently true over the range of light intensity for which they are used. If it is not the case, there may be apparent deviation from the theoretical relation between transmittance and concentration as defined by Beer's law (see p. 516), and such photometers are usually worthless for analytical purposes. Since the transmittancy is measured in a photoelectric photometer in terms of current output rather than of actual light intensity, it has been proposed that in such instances the term *photometric density* be used for the value of $-\log T_s$ rather than *optical density*. From a practical point of view this distinction is unimportant and will not be used here, but it serves to emphasize the point that the numerical value of the optical density for a given substance may be considerably influenced by the characteristics of the photometer used for the measurement.

Light absorption at specific wavelengths is an intrinsic property of many substances, e.g., hemoglobin and other colored compounds in the visible region of the spectrum, and many other compounds in the ultraviolet and infrared portions of the spectrum. It may therefore be used to characterize such substances, just as other physical constants are so used, in addition to its use in photometric analysis. The extinction at a specified wavelength for a unit amount of a particular substance in solution is known as the *specific extinction* or *extinction coefficient;* the amount is frequently defined as that present in a 1-cm. layer of solution containing 1 per cent of the substance (symbolized by $E^{1\%}_{1\text{cm.}}$). If the concentration is expressed on a molar basis, the term *molar* (or *molecular*) *extinction* is used. These constants are of more value for characterizing the substance in terms of its optical properties than for actual use in photometric analysis, but if they are sufficiently reproducible they may be used for the latter purpose, since they define the relationship between concentration and light absorption for the particular substance under specified conditions.

Beer's Law. The transmittance of a solution containing light-absorbing material depends upon (*a*) the nature of the substance, (*b*) the wavelength of the light, and (*c*) the amount of light-absorbing material in the light path, this latter depending in turn upon the concentration of substance and the depth of solution through which the light passes. The relation between these various factors was first clearly established for *colored* solutions by Beer, and hence is known as Beer's law, also as the Bouguer-Beer or Lambert-Beer law.[7] This relation may be expressed as follows: at a given wavelength,

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[7] For a fuller discussion of the laws of Bouguer and Beer, see Mellon: *Analytical Absorption Spectroscopy,* New York, John Wiley & Sons, Inc., 1950.

$$T_i = 10^{-k \cdot l \cdot c}$$

where T_i is the internal transmittance, k is a constant characteristic of the substance, l is the length or depth of solution through which the light passes, and c is the concentration of light-absorbing material. T_i is defined as the ratio I/I_o, where I_o is the radiant energy entering the sample and I that incident upon the second surface of the cell (see Fig. 126). The equation is exponential because of the particular characteristics of light absorption (see texts on physics for details); it may be converted to the more common logarithmic form by taking the logarithm of both sides, as follows:

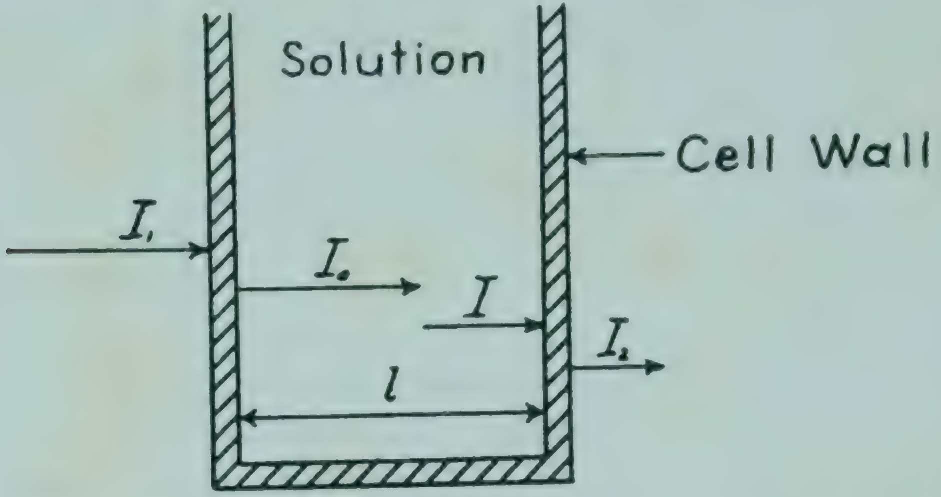

FIG. 126. SCHEMATIC DIAGRAM SHOWING DERIVATION OF TRANSMITTANCE (RATIO I_2/I_1).

Internal transmittance, T_i, is the ratio I/I_o; l is the length or depth of the absorbing path in the sample.

$$\log T_i = -k \times l \times c$$

or

$$-\log T_i = k \times l \times c$$

These equations relating transmittance, concentration, and depth of solution, at a given wavelength, are the fundamental ones upon which photometric analysis is based.

As mentioned on p. 512, for photometric purposes it is more practical to define transmittance in relative terms, i.e., the ratio of the (over-all) transmittance of the solution, T_{soln}, to that of the solvent (or reagent blank), T_{solv}, equal to T_s, the transmittancy. T_s is not precisely equal to $(T_i)_{soln}/(T_i)_{solv}$, but with end plates having a refractive index not greater than 1.5, the error is usually negligible.

Relation between Transmittance and Concentration. If the transmittances (in practice, transmittancy, T_s, values) of a series of solutions of a particular substance in various known concentrations are determined, at a particular wavelength and constant depth of solution (the usual conditions in a photometric analysis), the resultant data relating transmittance to concentration may be plotted in any one of four different ways, as shown in Fig. 127. If the transmittancy is plotted directly against concentration on ordinary cross-section paper, the curve of Fig. 127A is obtained. To obtain a straight line rather than a curve, and thus permit the accurate establishment of the relationship between transmittancy and concentration at only one or two concentrations instead of the many required for a curve, advantage is taken of the fact that in accordance with Beer's law the equation for the curve is:

$$\log T_s = -k' \times c$$

the constant depth of solution permitting the combination of l and k into k'. Thus if the *logarithm* of the transmittancy is plotted against concentration (Fig. 127B), or if transmittancy is plotted on the logarithmic axis of semilogarithmic paper (Fig. 127C), straight lines with negative slopes will result. Finally, if the Beer's law equation is written as follows:

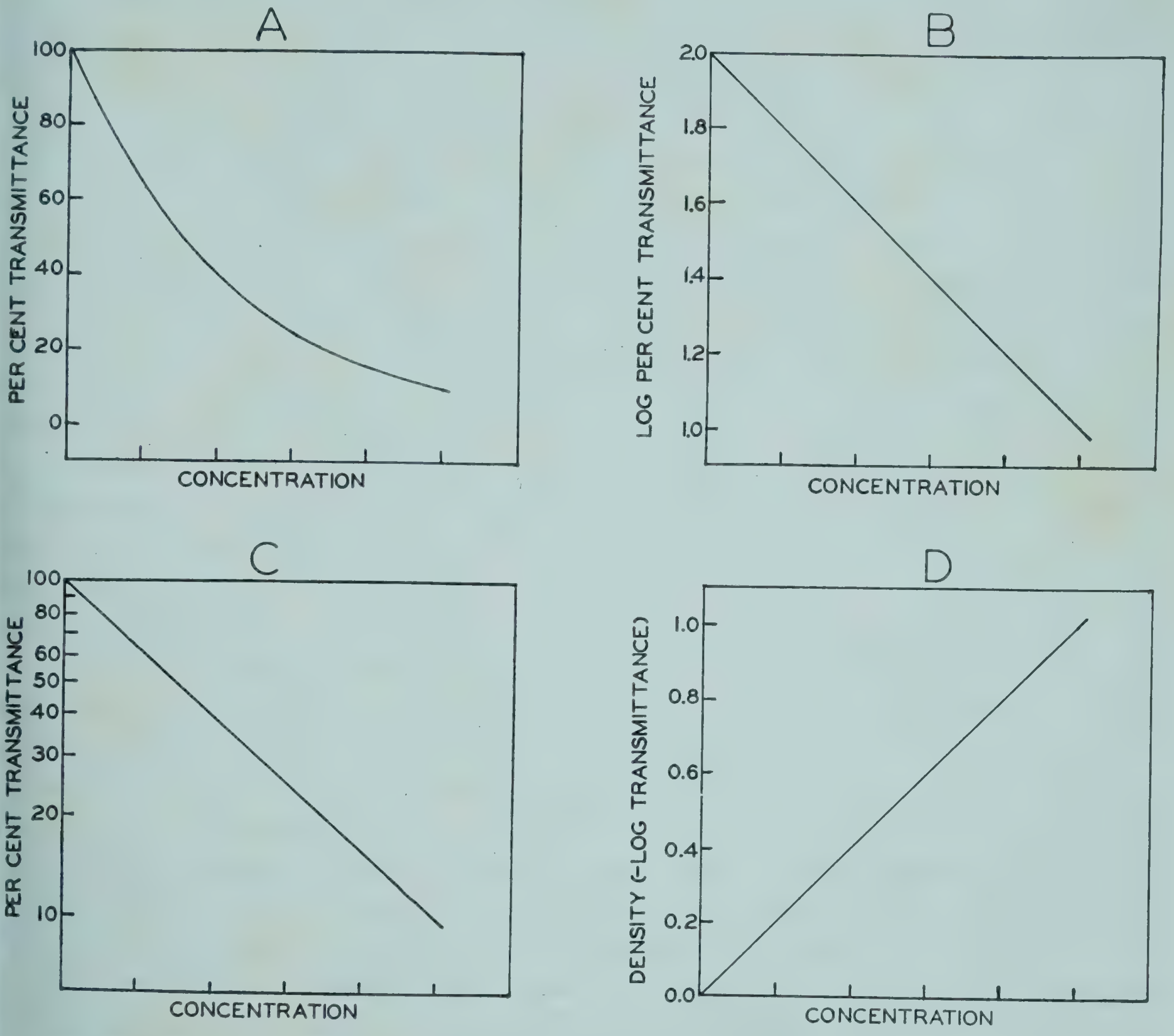

FIG. 127. VARIOUS WAYS OF SHOWING GRAPHICALLY THE RELATION BETWEEN LIGHT TRANSMITTANCE AND CONCENTRATION IN A PHOTOMETRIC PROCEDURE.

A, Per cent transmittance plotted directly against concentration. *B*, Log per cent transmittance against concentration. *C*, Per cent transmittance against concentration on semilogarithmic paper. *D*, Density (− log transmittance, or 2 − log per cent transmittance) against concentration.

$$-\log T_s = k' \times c$$

and the values of $-\log T_s$ plotted against c, a straight line with a positive slope results (Fig. 127D).

Of these four methods for relating transmittancy to concentration, the last is to be preferred. The quantity $-\log T_s$ has already been defined as the optical density, D, of the solution. Where Beer's law is applicable, the optical density is *directly proportional to concentration,* or

$$D = k' \times c$$

This represents the simplest possible relation between light absorption and concentration, and it is the major advantage obtained by expressing transmittancy in terms of optical density rather than as per cent.

The established relationship between transmittancy and concentration for a particular analytical procedure is known as the calibration for that procedure. If this relationship is shown in the form of a graph, as in Fig. 127, the graph is called the calibration curve. Every analytical procedure requires a separate calibration, over a specified range of concentration and at a given wavelength and depth of solution; and in general the calibration established for one photometer is not applicable to another photometer, even of the same make. The most accurate way to establish the calibration for a particular procedure, and the one which should be used whenever possible, is in terms of the reading of a simultaneously prepared standard (or, in some instances, series of standards) for that procedure, just as in accurate visual colorimetry, and for the same reasons. In practically all of the common colorimetric or photometric procedures, the substance being determined is subjected to a series of reactions leading to the final production of a color which is used as the basis for estimation. The many factors *aside from concentration* which influence the final color intensity in most if not all colorimetric procedures have been discussed on p. 501 in connection with colorimetric analysis; they are of equal if not greater importance in photometry. Furthermore, since photometric measurements are ordinarily made individually and in a sense "against the instrument itself," rather than by comparison against a standard color, variations in mechanical, optical, and electrical properties of the photometer may likewise influence the calibration. Thus the calibration for a particular procedure may vary from day to day, from one photometer to another, and from laboratory to laboratory. If calibration is established in terms of the reading of a simultaneously prepared standard at the time the analysis is carried out, the various factors other than concentration which may influence the actual readings exert the same effect on the standard and on the solution being analyzed, and thus do not influence the relative evaluation of one in terms of the other.

To calibrate a procedure in terms of the reading of a simultaneously prepared standard, advantage is taken of the fact that optical density (or extinction) is directly proportional to concentration where Beer's law is applicable, as it is to practically all photometric procedures under the proper conditions. Therefore, for two solutions of the same substance at different concentrations, at the same wavelength and depth of solution, the relation between their respective densities, D_1 and D_2, and concentrations, c_1 and c_2, is as follows:

$$\frac{D_1}{D_2} = \frac{c_1}{c_2}$$

If one of these two solutions is a standard of known concentration and the other is of unknown concentration, then if their respective densities are measured, the concentration of the unknown is given by the calculation:

$$\text{Concentration of Unknown} = \frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \begin{array}{c}\text{Concentration of}\\ \text{Standard}\end{array}$$

Thus the concentration of an unknown in a photometric procedure which shows agreement with Beer's law may be calculated from the determined density and the density of a known standard, and this method of obtaining results in a photometric analysis is used where applicable for all of the photometric procedures described in this chapter and elsewhere in this book.

The calculation is obviously quite similar to that used for visual colorimetric analysis with the Duboscq-type colorimeter (see p. 505), except for the direct instead of inverse proportionality between concentrations and readings. It is subject to the same limitations concerning the use of concentration rather than amount; if amount is used in the above equation rather than concentration, the unknown and standard must have the same total volume, otherwise the right-hand side of the equation is multiplied by V_X/V_S, where V_X is the total volume of unknown and V_S the volume of standard. To express results in terms of 100 ml. of blood, per liter of urine, or on some other arbitrary basis, the obtained result must be multiplied by a factor representing the relationship between the actual amount of sample used and the desired basis, as for colorimetric calculations.

To use this method of calculating results with photometers whose scale reads only in terms of per cent transmittancy, it is necessary to change the transmittancy value into its equivalent optical density. This may be done using a table of logarithms, or more simply by reference to the accompanying table, which gives the value of the optical density for all values of per cent transmittancy. Thus in an analysis, if the per cent transmittancy of standard and unknown are determined, readings are converted into density values by reference to the table, and results calculated as described above. If the photometer scale gives the density values directly, or reads in units which are proportional to density, the scale reading is used directly in the calculations. This is obviously more convenient, and a photometer scale of this type is to be preferred over one which is in terms of per cent transmittancy only.

Calculation of photometric results by the method described can of course be used only over the range of concentration where Beer's law is valid, i.e., where there is a linear relationship between optical density and concentration. For practically all photometric procedures in common use, conditions of wavelength, depth of solution, and concentration may be so selected that Beer's law is obeyed over the range of concentration apt to be encountered in an analysis. It is important in describing the details of a procedure to define these conditions, as has been done for the photometric procedures described in this chapter and elsewhere in this book.

RELATION BETWEEN TRANSMITTANCY (T_s) AND OPTICAL DENSITY (D)

T_s (%)	D	T_s (%)	D	T_s (%)	D	T_s (%)	D
100	0.000	75	0.125	50	0.301	25	0.602
99	0.004	74	0.131	49	0.310	24	0.620
98	0.009	73	0.137	48	0.319	23	0.638
97	0.013	72	0.143	47	0.328	22	0.658
96	0.018	71	0.149	46	0.337	21	0.678
95	0.022	70	0.155	45	0.347	20	0.699
94	0.027	69	0.161	44	0.357	19	0.721
93	0.032	68	0.168	43	0.367	18	0.745
92	0.036	67	0.174	42	0.377	17	0.770
91	0.041	66	0.181	41	0.387	16	0.796
90	0.046	65	0.187	40	0.398	15	0.824
89	0.051	64	0.194	39	0.409	14	0.854
88	0.056	63	0.201	38	0.420	13	0.886
87	0.061	62	0.208	37	0.432	12	0.921
86	0.066	61	0.215	36	0.444	11	0.959
85	0.071	60	0.222	35	0.456	10	1.000
84	0.076	59	0.229	34	0.469	9	1.046
83	0.081	58	0.237	33	0.482	8	1.097
82	0.086	57	0.244	32	0.495	7	1.155
81	0.092	56	0.252	31	0.509	6	1.222
80	0.097	55	0.260	30	0.523	5	1.301
79	0.102	54	0.268	29	0.538	4	1.398
78	0.108	53	0.276	28	0.552	3	1.523
77	0.114	52	0.284	27	0.569	2	1.699
76	0.119	51	0.292	26	0.585	1	2.000

On photometers equipped with a linear scale reading from 0 to 100, D corresponds to the value of $2 - \log G$ where G is the galvanometer or microammeter reading relative to an initial setting at the 100 mark.

For a few procedures, of which the determination of blood creatinine by the alkaline picrate reaction (p. 555) is an example, it is found that Beer's law is not followed under any analytical conditions. In such cases a calibration curve must be constructed and results obtained by reference to the curve as described below; for the most accurate results such a curve should be constructed with each series of analyses unless it is found to be highly reproducible. Instances of deviation from Beer's law are fortunately very rare. It is more common to find an apparent deviation from Beer's law when agreement is expected. This is usually the fault of the photometer. In most instances, agreement with Beer's law can be expected only when essentially monochromatic light is used, particularly when photovoltaic cells with their inherent varying spectral sensitivity are used in the photometer. If a wide rather than a narrow portion of the spectrum is used for measurement, as is unfortunately the case with some types of photometers, or if the photocell circuit is such that current output is not proportional to light intensity, the relationship between optical density (or $-\log T_s$) and concentration will not be linear, and results must be based upon a calibration curve. Photometers with such characteristics are therefore undesirable as a basis for accurate photometric analysis, because they do not permit the analytical precision associated with the application of Beer's law to the procedure.

Another commonly used method for obtaining results in a photometric analysis is based upon the use of previously prepared graphs of the type shown in Fig. 127, or of tables based upon the graphical data. The graph or table is established for a particular procedure by determining the transmittancy values for a sufficient number of solutions of known and varying concentration with respect to the substance being determined. In future analyses, the transmittancy of the solution of substance in unknown concentration is determined, and its concentration is then found from the graph or table, without running a standard at the same time ("colorimetry without standard solutions"). It is not necessary that the color reaction show agreement with Beer's law, since the relation between photometer reading and concentration is established empirically; this type of procedure therefore finds its greatest use where for one reason or another there is lack of agreement with Beer's law. An analogous procedure in instances where Beer's law is valid is to establish the density of a known standard and to use this value in future analyses, i.e., the concentration of the unknown is obtained by multiplying its determined density by a factor representing the established relationship between the standard and its density (see the photometric determination of hemoglobin, p. 610, for examples). The extinction coefficient of a substance may also be used in a similar way.

It is assumed in the use of such previously obtained calibration data that a particular transmittancy or density will always represent a particular concentration in an analysis. In practice this may or may not be true. The many factors which influence color intensity aside from concentration have already been emphasized. Even for substances such as hemoglobin in which the light-absorbing power is an integral property of the molecule itself, variations in environmental conditions or in the photometer itself may influence readings. Thus the use of a previously prepared calibration curve may be at best only an approximation, and gross errors are known to have resulted from its use. Colorimetry without standard solutions does not exist; the use of a previously prepared calibration curve simply represents a decision that the standard is to be prepared and read at one time and the unknown at some other time and possibly under different conditions, rather than that standard and unknown are to be prepared and read under the same conditions. It may be stated without equivocation that the greater desirability of the latter procedure has never been seriously challenged.

It is true that in some instances, as where the standard substance is difficult to obtain or maintain stable in solution, or the scope of the analytical problem permits the sacrifice of accuracy to convenience, a calibration curve may satisfy analytical requirements. If such a curve is used, it should be constructed in one's own laboratory using the reagents and photometer which will actually be employed in the analysis. Calibration data obtained from the literature or from the manufacturer of the photometer should never be used without checking, and this checking must be repeated at frequent intervals if satisfactory results are to be expected, particularly if there has been a change in the reagents or photometer. The analyses must be carried out with rigorous control of the various steps involved, reproducing as far as possible the conditions under which the curve was constructed. Only in this way can the occurrence of serious errors be prevented.

Relation between Transmittancy and Wavelength. The relation between the transmittancy (and hence the optical density) of a solution containing light-absorbing material and the wavelength of light passing through the solution is given by the so-called absorption spectrum of the substance. The absorption spectrum is established quantitatively by measuring the transmittancy for a particular concentration and depth of solution at various wavelengths, and plotting the results in the form of a

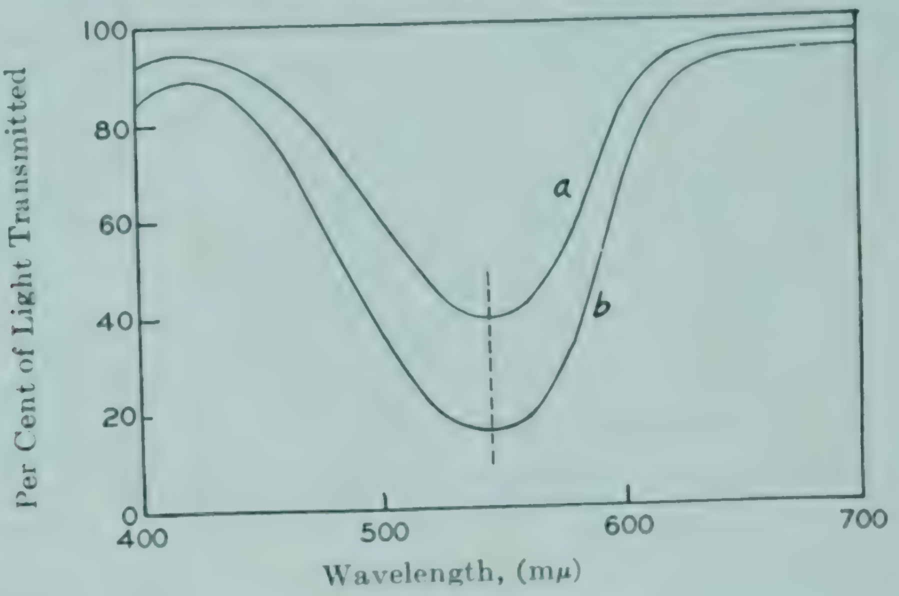

FIG. 128. A RELATIVELY SIMPLE ABSORPTION SPECTRUM.

Curve *b* represents a concentration twice as great as for curve *a*. The dotted line indicates the wavelength of maximum sensitivity for photometric measurement.

curve relating transmittancy or optical density (the latter is preferred) to wave-length. An example of a relatively simple absorption spectrum is shown in Fig. 128; more complex curves are frequently found. The absorption spectrum of a substance is usually characteristic of the substance and may serve for identification as well as furnishing information of analytical value. The application of absorption spectra is not limited to the visible region of the spectrum but may be applied equally well to characterization of the ultraviolet or infrared absorption of many substances.

For a particular substance, absorption curves at different concentrations will be generally similar in shape but will differ in their position along the transmittancy axis (curves *a* and *b* in Fig. 128). It will be noted that for a particular substance at a given concentration, there may be a certain amount of light absorption (transmittancy less than 100 per cent) at most wavelengths, but that this absorption is relatively greater in some spectral regions than in others. In the visible region of the spectrum, this difference in relative light absorption at various wavelengths is of course the cause of *color*, since when white light containing all wavelengths passes through the solution, the emergent light contains a greater proportion of some wavelengths than of others, and hence appears colored.

As the concentration of substance in solution is increased (Curve *b* relative to Curve *a*) there is usually increased light absorption at all wavelengths where any light is absorbed at all, but this increase is usually greater per unit change in concentration at some wavelengths than at others. Thus the wavelength used may determine the relationship between concentration and optical density at a constant depth of solution (i.e., the numerical value of *k* in the Beer's law equation), and this is frequently a major factor in the choice of the proper wavelength for photometric measurement.

Maximum sensitivity in a photometric procedure is obtained at the wavelength where there is the greatest change in optical density or transmittancy per unit change in concentration. Most often, this is the wavelength of maximum light absorption, or *minimum* in the transmittancy curve (dotted line, Fig. 128). Occasionally, however, as for example in a procedure where the transmittancy curves for reagent and colored compound overlap considerably, the highest sensitivity may be obtained at a wavelength on one side of the absorption maximum. Thus, a knowledge of the absorption spectrum of both reagent and colored compound (the latter at two or more concentrations) is necessary for selection of the optimum wavelength.

Maximum sensitivity is not *per se* the chief consideration in the selection of the proper wavelength for photometric measurement. From an analytical point of view, the most satisfactory wavelength is the one which, at a given depth of solution, shows agreement with Beer's law over as wide a range as possible of the concentrations apt to be encountered in an analysis, and which permits this range to be read within the most accurate region of the photometer scale. The most accurate region of the scale corresponds to densities between about 0.2 and 1.0 (60 to 10 per cent transmittancy). Readings outside this range represent solutions which are either too light or too dark for the most accurate measurement; thus at 95 per cent transmittancy an absolute error of 0.5 per cent in the transmittancy measurement corresponds to a 10 per cent error in an analysis; and at the other end of the scale, for dark solutions unit change in transmittancy represents a disproportionately large change in concentration. The sample should therefore read between the scale limits specified if maximal accuracy is to be obtained.

To fulfill these requirements in the case of the Folin-Wu blood sugar method, for example (see p. 568), a wavelength may be selected which represents a very low sensitivity, so that the normal glucose standard representing 100 mg. per cent blood sugar will have a low light absorption. This would permit reading blood sugar values well above normal, which is the usual direction of change in this procedure, under the same conditions. If the usual direction of change is below the normal value, as in hemoglobin determinations, conditions are selected such that the normal sample has a high light absorption. In general, to adapt to photometric measurement a procedure which was originally developed for visual colorimetry and hence may yield a more intense color than is required for photometric measurement, and in a larger volume of solution, it is better to modify the light absorption. This may be done by selection of a suitable wavelength or use of a small depth of solution rather than by such procedures as taking a smaller sample or diluting the final color, since these may seriously affect the accuracy. However, the greater sensitivity of photometric measurement, and the possibility of using very small volumes of solution, is obviously conducive to the development of microanalytical methods, of which many have been and are being developed, and this represents an important contribution of photometry to analytical chemistry.

Other factors which may influence the choice of wavelength for photometric measurement include the possibility that agreement with Beer's law will be found to be more satisfactory over a wider range of concentration at one wavelength than at another, or that the color will be more stable when exposed to light of one wavelength than to that of another. Even if such selection entails a decrease in sensitivity, the enhancement of the analytical value of the procedure may make the wavelength of lesser sensitivity the one of choice. If two or more light-absorbing substances are present together, and it is desired to measure the change in optical density related to variation in amount of only one of these substances, it is sometimes possible to select a wavelength at which there is only minimal light-absorption by extraneous material, and thus prevent such material from interfering significantly in an analysis. Such photometric separation is rarely complete; it is usually better to effect preliminary analytical separation, or to include the light absorption from extraneous material in the blank solution used for the initial setting of the photometer.

Knowledge of the complete spectral characteristics of a light-absorbing compound is of fundamental importance in defining the conditions under which satisfactory photometric analysis is possible. Wherever possible such information should be made available in the description of a photometric procedure, as has been done in many instances for the various photometric procedures described in this chapter and elsewhere in this book. The detailed description of the spectrophotometric characteristics of the Nessler reaction with ammonia, given in Chapter 31, illustrates the applicability of absorption spectrum data to photometric procedures.

Light Filters and Filter Photometers. The wavelength at which photometric measurements are made may be established either by the use of light filters or by the production of a complete spectrum and isolation of the desired portion. Instruments based upon the first principle are known as filter photometers; those based upon the second principle are called spectrophotometers (see p. 531). Most types of photometers in current use are filter photometers, though spectrophotometers are now extensively used, even for routine analyses. Filter photometers, however, are less expensive than spectrophotometers, require less technical skill in operation, and if well designed are just as satisfactory for most analytical purposes, particularly in the visible region of the spectrum. Except for special instances, spectrophotometers must ordinarily be used for photometry in the ultraviolet and infrared portions of the spectrum.

Light filters commonly consist of selected glass (or sometimes dyed gelatin) which is capable of transmitting light over a limited portion of the spectrum only. Thus by placing such a filter in the light path of the photometer, measurements may be made in the spectral region corresponding to the transmittance range of the filter. Various filters differ principally with regard to (*a*) the spectral region of light transmittance, and (*b*) the width of the transmitted band. By suitable selection of various types or combinations of glass, it is usually possible to obtain a filter whose transmittance is limited to almost any desired portion of the spectrum. A selection of such filters is illustrated in Fig. 129. Suitable filters may be obtained from the manufacturers of the photometer, or they may be constructed in the laboratory if suitable glass is available. It is customary to designate a filter in terms of the wavelength of peak transmittance, thus a filter called "No. 540" or "No. 54" has its peak transmittance at a wavelength of 540 mμ. This practice is not universal, nor is the wavelength designation always accurate, and when a filter of any kind is used in a photometric procedure the wavelength of peak transmittance should be stated also. If this information is not known, it may usually be obtained from the manufacturer of the filter or glasses used. When the wavelength required for a particular photometric procedure is stated in the description of the procedure, as for example, "at 540 mμ," this corresponds to the use in a filter photometer of a filter with a peak transmittance at this wavelength.

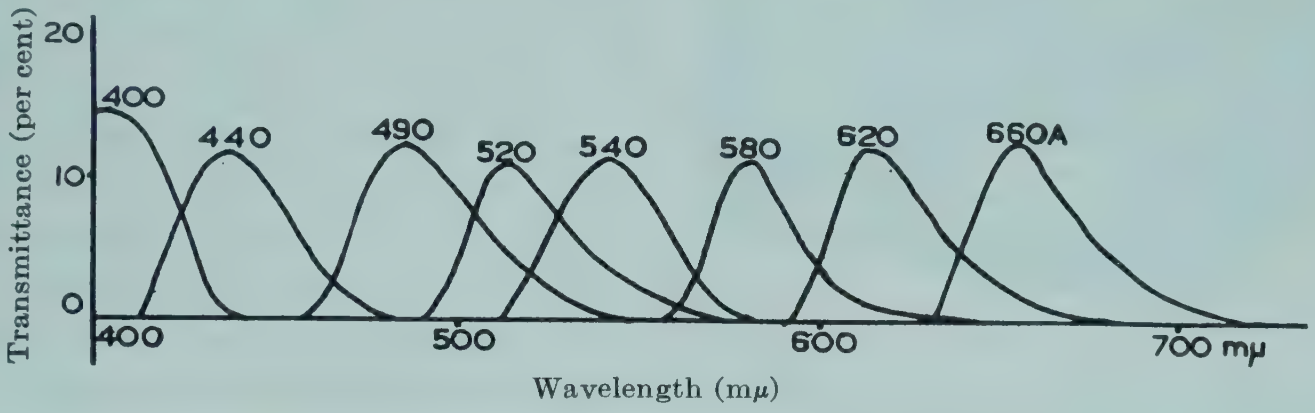

FIG. 129. LIGHT TRANSMITTANCE OF SELECTED LIGHT FILTERS.
Courtesy, Rubicon Co.

The most satisfactory filters are those which transmit as narrow a spectral region as possible, since this represents an approach to truly monochromatic light. A good filter for photometric purposes will show a transmittance of about 85 per cent or more of the total light transmitted over a spectral width of 30 to 60 mμ or so, centered around the wavelength of peak transmittance. This information concerning a filter may be obtained from its transmittance curve, as illustrated in Fig. 129. Filters with a broader range of transmittance are in general unsatisfactory, because they may result in apparent deviations from Beer's law, as discussed on p. 520.

In recent years interference filters (metallic films on glass or fused quartz) have come into use, such as, for example, those made by Baird, Farrand, and others. The Farrand filters consist of evaporated thin layers of dielectric material between semitransparent metallic films on glass.

Interference filters are sometimes called *monochromatic filters* because of their band pass of only 20 mμ or less, much narrower than the usual glass or gelatin filters. These filters, however, are not monochromatic in the sense that light of a single wavelength is monochromatic, since they transmit a narrow range of wavelengths rather than a single wavelength. Interference filters have a high transmission, being about 40 per cent—more than twice that possible with ordinary filters of equal band pass.

Representative types of filter photometers commercially available are illustrated in Figs. 130, 131, and 133. Many other types are on the market,[8] some of which are doubtless as satisfactory as those illustrated. The Hellige "Chromatron" (Fig. 130) is an example of the single photocell type of photometer, with built-in meter, the scale of which reads linearly from 0 to 100, and hence gives transmittancy in per cent. This instrument is designed for use with rectangular pyrex cuvettes. The light source is mounted in a self-focusing housing and is operated below its rated wattage either from two dry-cell batteries or from an A.C. line through a built-in constant voltage transformer. The filters are mounted in a rotating disk close enough to the photocell to avoid interference from stray light. The photocell is hermetically sealed in an inert atmosphere. In operation, the instrument is adjusted to a scale reading of 100 with water and a suitable filter in place; the water is then replaced by the solution under examination, and the scale reading noted. This value gives the transmittancy of the sample, in per cent. To convert transmittancy into optical density, the value of 2 − log *R* is obtained, where *R* is the scale reading, or the table on p. 520 may be used. Users of this type of instrument appear to prefer the calibration curve method of obtaining results, rather than calculation based upon optical density. The Hellige instrument is also available in a model ("Clinicol") precalibrated for a variety of clinical procedures which are described in an accompanying handbook.

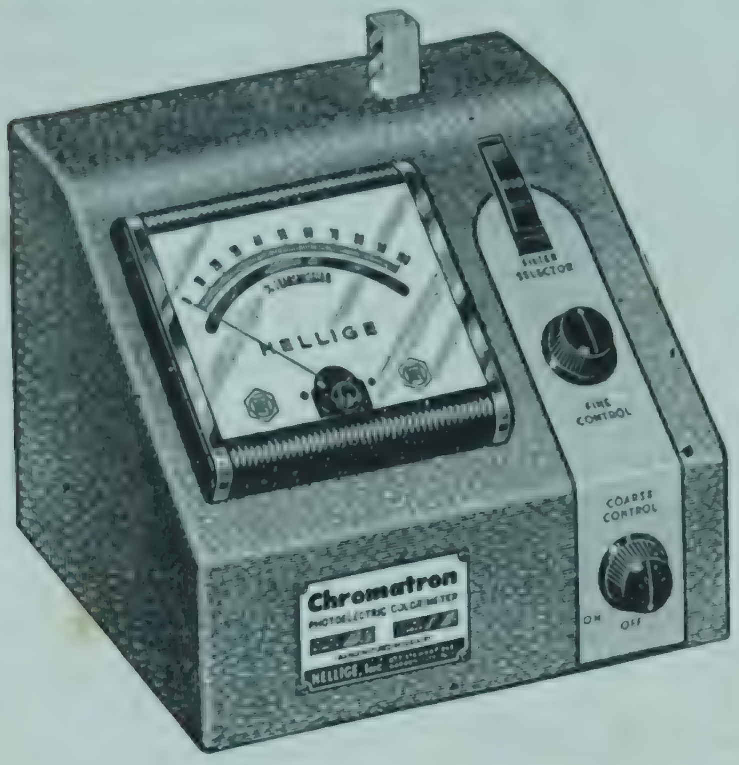

FIG. 130. HELLIGE "CHROMATRON" PHOTOELECTRIC COLORIMETER.
Courtesy, Hellige, Inc.

The Evelyn photoelectric colorimeter (Fig. 131) is likewise a single photocell photoelectric filter photometer, with uniform test tubes customarily employed as solution containers, and with readings made on a sensitive galvanometer which is separated from the rest of the instrument. The galvanometer scale is graduated linearly, from 0 to 100, so that readings here are also in terms of per cent transmittancy. A diagram illustrating the schematic construction of the Evelyn instrument is shown in Fig. 132. With a suitable filter in place, the reference fluid in a special test tube is placed in the instrument and the light intensity adjusted by resistance control until the meter reads 100. The reference fluid is then replaced by the sample, in a second similar test tube, and the galvanometer reading noted. Its value gives the per cent transmittancy of the sample. To convert transmittancy into optical density, the value of $2 - \log G$ is obtained, where G is the galvanometer reading; or the table on p. 520 may be used.

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[8] Manufacturers of satisfactory photometers include Central Scientific Co., Chicago; Rubicon Co., Philadelphia; Klett Manufacturing Co., New York; Fisher Scientific Co., Pittsburgh; American Instrument Co., Silver Springs, Md.; Coleman Instruments, Inc., Maywood, Ill.; Hellige, Inc., Garden City, N. Y.; Photovolt Corp., New York; Pfaltz and Bauer, New York; Bausch and Lomb Optical Co., Rochester; and others. Some of these concerns likewise manufacture fluorimeters. Information concerning various types available usually may be secured from any laboratory supply house.

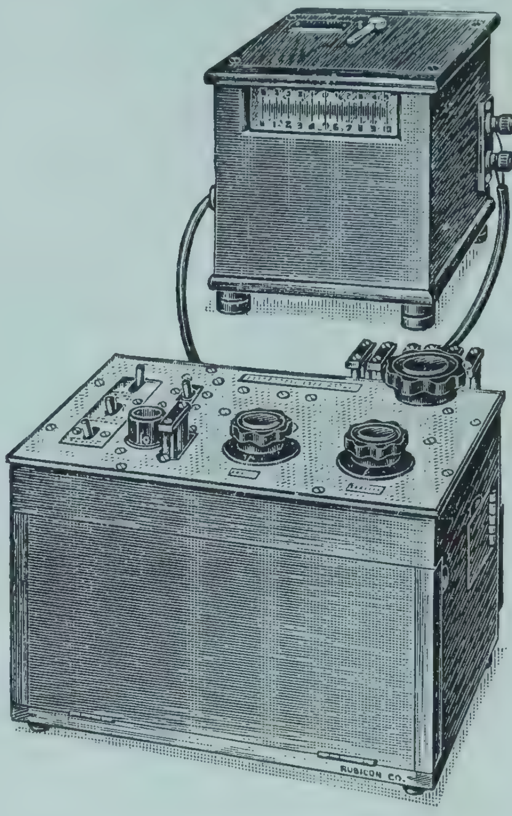

FIG. 131. EVELYN PHOTOELECTRIC COLORIMETER.
Courtesy, Rubicon Co.

The light source is a 6-volt bulb, operated from a storage battery to provide constancy of illumination, which is essential with all single cell photometers. The filters used with this instrument are particularly satisfactory from the point of view of narrowness of spectral band; the selec-

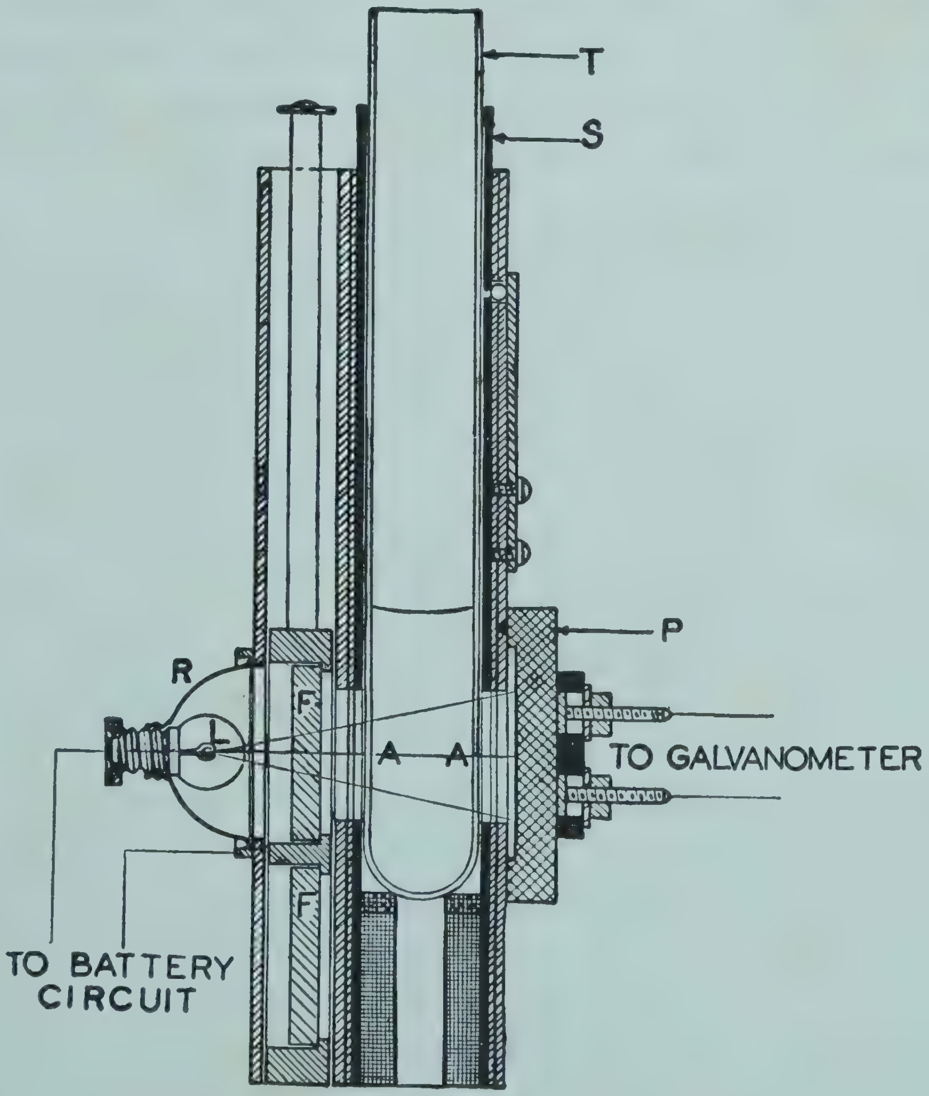

FIG. 132. SCHEMATIC DIAGRAM OF EVELYN PHOTOELECTRIC COLORIMETER.

A, Apertures; *F*, Filter; *L*, lamp; *R*, reflector; *S*, shield; *T*, test tube; *P*, photocell.

tion available is shown in Fig. 129. The test tubes require a minimum of about 6 ml. of solution for a reading; a microcolorimeter, requiring much less fluid, is also available for use with the instrument. The use of test tubes as solution containers or cuvettes has the great advantage that many colorimetric procedures may be carried out partially or wholly in the same tube as will be used for the final reading. The Evelyn test tubes have dimensions such that they correspond roughly to a 2-cm. solution thickness, so that the optical density for a particular concentration of substance will be about twice that expected at 1-cm. depth, which is the usual basis of reference, and which is used throughout this chapter. This means that in the Evelyn instrument, a sensitivity and concentration range specified for 1-cm. solution depth will correspond to about twice the sensitivity, over the lower half of the specified concentration range only; higher concentrations will prove to be too dark to read accurately. This must be considered in interpreting data obtained by this instrument or for use with it.

The Bausch and Lomb "Monochromatic" Colorimeter is a single-photocell, direct-reading photoelectric colorimeter, equipped with narrow-band interference filters. (See p. 525.) A 6-volt, 32-c.p. incandescent lamp, operated from a constant voltage transformer, supplies the light for both the scale and the analysis path. Light from the lamp passes through condensing lenses, then through a heat-absorbing filter, past a control diaphragm, through an interference filter, and then through the sample solution, finally striking a barrier-layer photocell. The current output of the photocell is measured by a sensitive double-suspension galvanometer, the deflection of its mirror (indicated by a floating spot-light) being measured upon the translucent scale at the front of the colorimeter.

Advantages of single-photocell photometers such as the three just described include the simplicity of construction and the fact that readings are made on a direct-reading meter which does not require manual adjustment. Thus even relatively unstable colors, such as that obtained in the antimony trichloride reaction for vitamin A, may be read immediately or at successive small time intervals for extrapolation. The major disadvantage of the single-photocell type is the requirement for a stable light source, to eliminate the possibility of error due to a change from the initial light intensity during a measurement. To provide a constant current for the light source, storage batteries or constant-voltage transformers are used; the former is generally more satisfactory, since the efficiency of constant-voltage regulators may depend upon the type of power supply available. Another disadvantage of single-photocell photometers is that they usually incorporate meters designed primarily for current measurement and not for photometric purposes, with linear graduation and frequently a small total scale length, which may make precise reading difficult. Meters should be accurately readable to one-third of a scale division, for example, if a reading error of 1 per cent or less is expected over one-third of the scale range. Linear graduation of the scale requires the use of logarithms or conversion tables to convert per cent transmittancy values into optical density, which is a much more satisfactory basis for photometric analysis. Some types of single-cell photometers are equipped with scales reading in terms of optical density as well as, or rather than, per cent transmittancy; it is not unreasonable to hope that this practice will be extended.

To overcome some of the disadvantages of single photocell photometers, various types of photometers employing two photocells in a balanced circuit have been developed, of which the Klett-Summerson instrument (Fig. 133) is an example. The details of construction of this instrument are shown in Fig. 134. In operation, with a suitable filter in place, the reference solution contained in a test tube is placed in the path of light striking one of the two photocells, which are arranged in a potentiometric circuit so that the current from one cell is opposed to that from the other through a null-point instrument (low-sensitivity galvanometer). With the photometer scale set at zero (corresponding to zero optical density) the current output from the second photocell is adjusted so that it exactly balances that coming from the photocell which is subject to the light emerging from the solution. This balance is indicated by a zero reading on the galvanometer. The reference solution is then removed and replaced by the solution under examination. Any light absorption by this solution will throw the two photocells out of electrical balance; balance is then restored by turning the potentiometer dial until the galvanometer again reads zero. The reading on the potentiometer scale at this point is the measure of the light absorption of the solution.

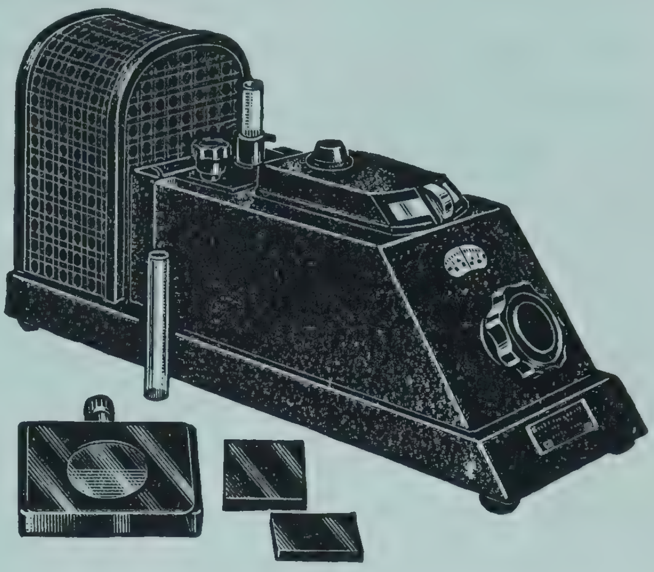

FIG. 133. KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER.
Courtesy, Klett Manufacturing Co.

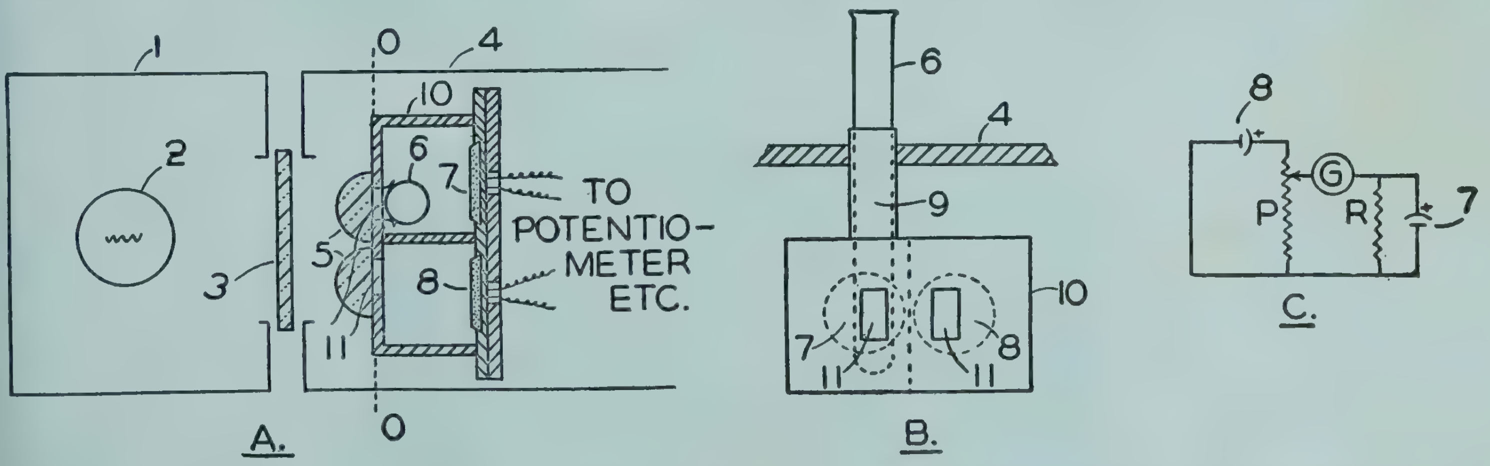

FIG. 134. DIAGRAMMATIC DETAILS OF KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER.

A. Schematic view of the rear half of the instrument, in section from above. *B*. View across the line 00 in *A*, looking toward the front of the instrument. *C*. Wiring diagram of the photoelectric cell circuit. *1*, Lamp housing; *2*, lamp; *3*, light filter; *4*, instrument housing; *5*, compensating lenses; *6*, test tube; *7*, "working" photoelectric cell; *8*, "reference" photoelectric cell; *9*, metal tube (light shield); *10*, photoelectric cell compartment housing; *11*, light slits in compartments; *P*, 400-ohm potentiometer; *R*, 400-ohm fixed resistance; *G*, low-sensitivity galvanometer.

Summerson, *J. Biol. Chem.*, **130**, 149 (1939).

The light source is a 100-watt bulb operated directly from an ordinary power supply; the balanced circuit prevents fluctuations in light intensity from influencing readings. The instrument is designed for use with light filters of relatively narrow spectral transmission; the selection available is similar to that shown in Fig. 129. The test tubes require about 5 ml. of solution for a reading; microtubes requiring about 2 ml. may also be used. The test tubes may be used for color development as well as for reading, and may be centrifuged if necessary. The effective solution depth is approximately 1 cm., so that photometric data based on this depth of solution are directly applicable to the instrument. Models permitting the use of glass cells at other solution depths are also available.

The scale on the Summerson instrument deserves comment, because it is somewhat unusual. It is graduated in units which are proportional to optical density; the actual numerical values represent the optical density divided by two and with the decimal point omitted. Thus a scale reading of 250 corresponds to an optical density of 0.500; of 100, to 0.200, and so on. In general, the relation between scale reading R and optical density D is as follows:

$$\frac{1000 \times D}{2} = R$$

Thus the fractional values of optical density (see table on p. 520) have been replaced by whole numbers, to facilitate use in photometric calculations. Since the scale readings bear a constant relation to optical density, they may be used directly in place of density values in the calculations of photometric analysis.

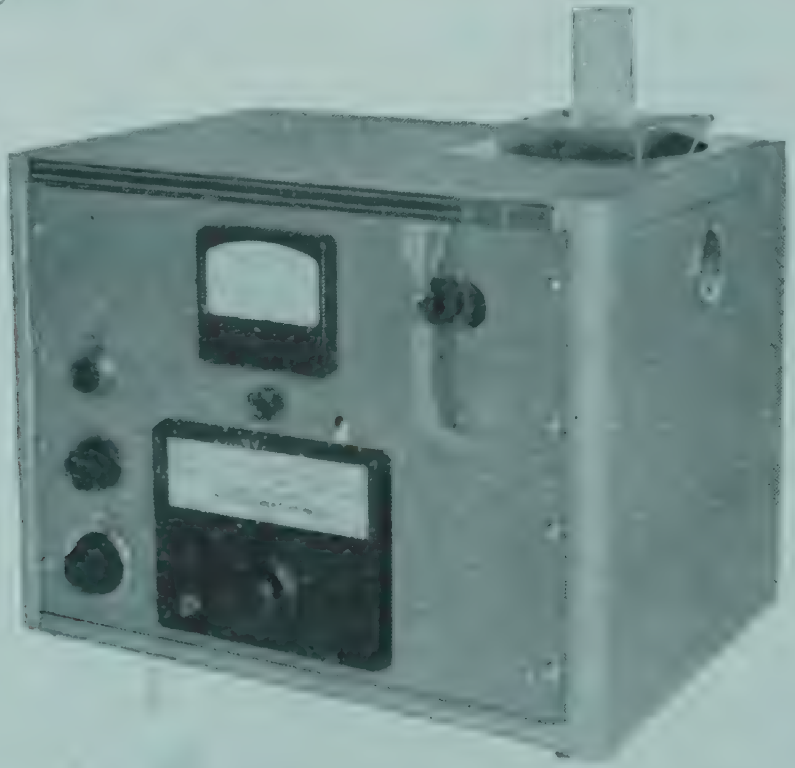

FIG. 135. PERKIN-ELMER FLAME PHOTOMETER.
Courtesy, Perkin-Elmer Corp.

An interesting recent development in filter photometry is the flame photometer (Figs. 135 and 136).[9] This was designed primarily to facilitate the rapid and accurate analysis of such metallic elements as sodium and potassium, which have intense and characteristic flame spectra. The sample in solution is blown in the form of a fine spray into a colorless flame, and the intensity of the resulting flame spectrum is determined by the response of a photoelectric cell, as measured on a galvanometer or microammeter. The instrument is calibrated for a particular metal by running a series of standards containing varying concentrations of the metal ion. No ashing or other preliminary preparation of the sample is ordinarily required; sodium and potassium determinations on blood plasma or serum for example require merely appropriate dilution with water. This instrument is known to give excellent results, and has proved to be quite valuable in the particular analyses for which it is fitted.

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[9] For a review of flame photometry, see Barnes, Richardson, Berry, and Hood: *Ind. Eng. Chem. Anal. Ed.*, 17, 605 (1945). Also Berry, Chappell, and Barnes: *Ind. Eng. Chem. Anal. Ed.*, 18, 19 (1946).

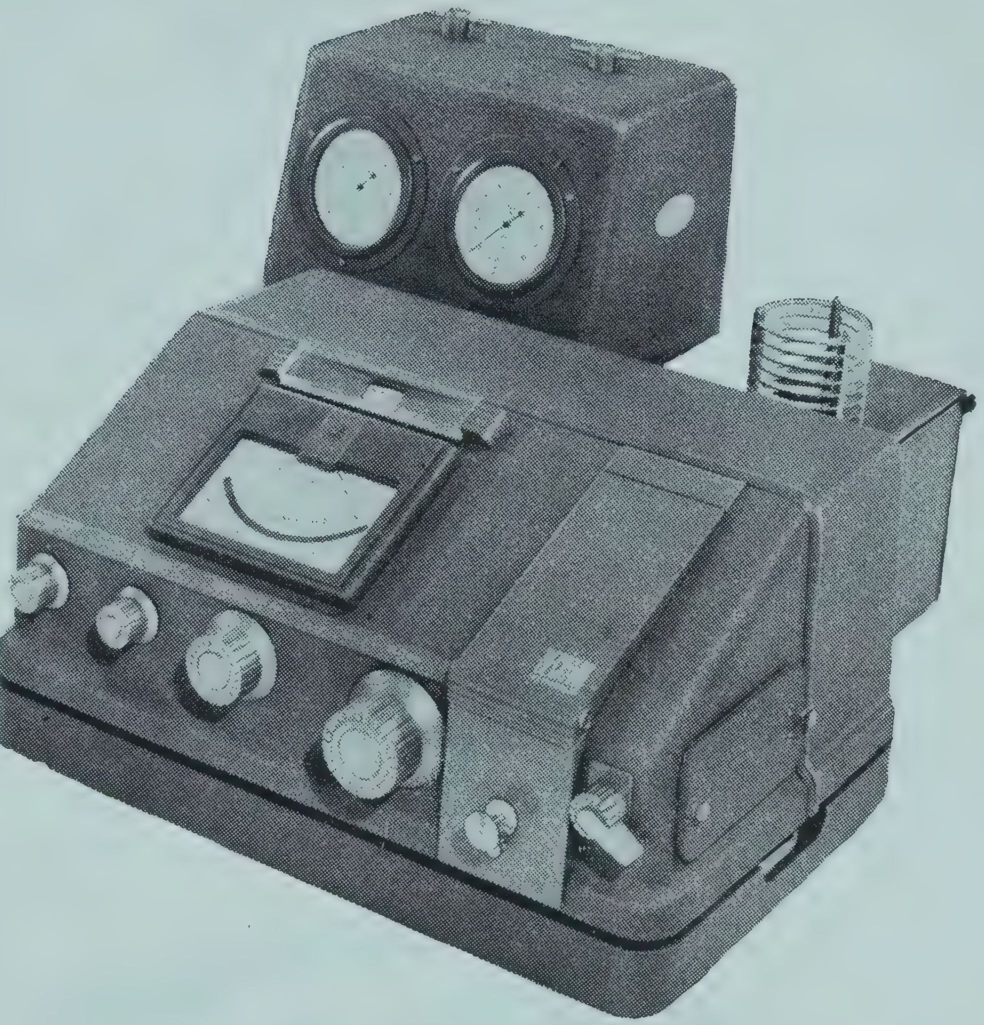

FIG. 136. BECKMAN MODEL B SPECTROPHOTOMETER WITH FLAME PHOTOMETRY ATTACHMENT.

Courtesy, Beckman Instruments, Inc.

Spectrophotometers. In place of using light filters for isolating the narrow spectral region usually required for photometric analysis, a device for producing the complete light spectrum and isolating the desired portion may be used. Instruments based upon this principle are known as spectrophotometers.[10] In the Beckman photoelectric spectrophotometer (Figs. 137 and 138), the spectrum is produced by the use of a quartz prism; a diffraction grating may also be used, as in the Coleman instrument (Fig. 139). The spectral band is focused on a narrow slit mounted in front of the solution being examined, and by shifting the band across the plane of the slit the desired spectral region is obtained. Spectrophotometers are usually more expensive than filter photometers, the cost increasing with increased sensitivity, spectral range, and narrowness of the spectral region isolated.

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[10] Manufacturers of spectrophotometers include Beckman Instruments, Inc., South Pasadena, Calif.; Coleman Instruments, Inc., Maywood, Ill.; and Central Scientific Co., Chicago. Information may also be obtained from the larger laboratory supply houses.

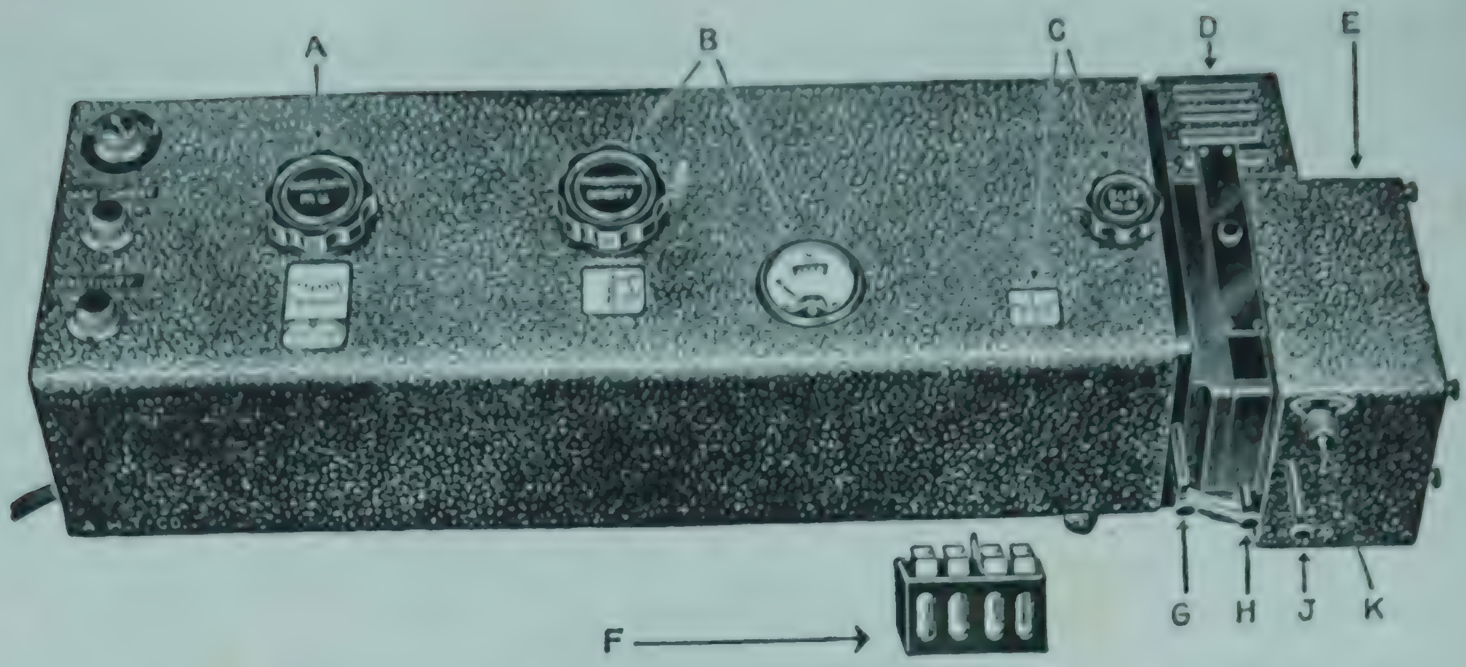

FIG. 137. BECKMAN PHOTOELECTRIC QUARTZ SPECTROPHOTOMETER.

A, Wavelength scale; *B*, built-in electronic indicating meter; *C*, slits with precision adjustment; *D*, light source; *E*, compartment for two phototubes; *F*, holder for four 10-mm. absorption cells; *G*, filter slide; *H*, compartment for absorption cells; *J*, phototube selector; and *K*, switch for checking dark current.

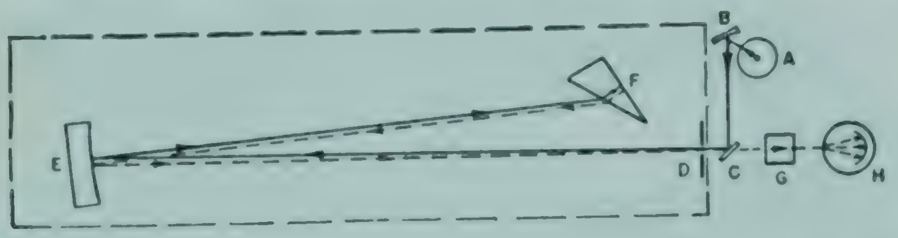

FIG. 138. SCHEMATIC DIAGRAM OF BECKMAN SPECTROPHOTOMETER.

A, Light; *B*, *C*, and *E*, mirrors; *D*, slit; *F*, prism; *G*, cuvette; *H*, phototube.

Courtesy, Cary and Beckman: *J. Opt. Soc. Am.*, **31**, 682 (1941).

FIG. 139. COLEMAN MODEL 6A JUNIOR SPECTROPHOTOMETER.

Courtesy, Coleman Instruments, Inc.

For routine analytical purposes, particularly in the visible region of the spectrum, spectrophotometers have the advantage over filter photometers of greater convenience and flexibility in choice of wavelength; this is offset to a certain extent by the increased cost as compared to filter photometers of equal or greater accuracy, and by the increased technical skill required in operation and maintenance. The wavelength setting is an extra adjustment connected with each analysis; variation or alteration is obviously more likely to occur than if a stable glass filter of suitable optical characteristics is used for controlling the spectral range. Errors due to inaccurate wavelength setting may be minimized in an analysis if results are obtained in terms of a standard prepared and read under the same conditions as for the unknown, but in any event frequent checking of the accuracy of the wavelength setting is important. The major use of spectrophotometers in photometric analysis at the present time is in connection with substances whose light absorption is in the ultraviolet or infrared regions of the spectrum, since no other type of instrument can be used for this particular purpose, and for the establishment of the complete spectral characteristics of a color (i.e., the absorption spectrum).

The Beckman quartz spectrophotometer is especially adapted to measurements in the ultraviolet and has had wide application in the study of vitamin A, sterols, hormones, etc. An infrared model is also available.

Choice of Photometers. The choice of photometers from the many types commercially available is largely a question of the requirements of the individual laboratory. For routine analytical purposes based upon established procedures, any good type of filter photometer will prove satisfactory. Instruments equipped for the use of test tubes as solution containers are preferable to other types, because of the convenience and low cost of this type of cuvette; in some laboratories, however, measurements may have to be made at varying depths of solution, and an instrument capable of being used with different sizes of containers will be required. If a single-photocell type instrument is desired, the stability on the laboratory current should be tested before use, or facilities for maintaining a storage battery must be available. Filter photometers differ considerably in the width of spectral band transmitted by the filters supplied with the instrument; in general, the narrower the band the more satisfactory will be the photometer; agreement with Beer's law cannot be expected with filters transmitting a wide range of wavelengths. For analytical purposes, a spectrophotometer covering only the visible and neighboring portions of the spectrum is little better than a good filter photometer, except for the convenience of wavelength selection. For investigational purposes the spectrophotometer should be usable in the ultraviolet and near infrared regions as well as in the visible region; an ideal combination would include such a spectrophotometer for investigational purposes, and a good filter photometer for analytical purposes.

Turbidimetry and Nephelometry. The light transmittance of a fluid is influenced not only by the amount of light-absorbing material present in solution but also by the presence of light-scattering or light-obstructing material such as insoluble substances in suspension. Quantitative analysis of substances in suspension based upon this principle is known as turbidimetry or, less commonly, nephelometry. These two terms are substantially equivalent, although nephelometry is usually considered to include the use of the intensity of scattered light (i.e., light at right angles to the incident beam) as a measure of turbidity, as well as methods based on transmittance measurements. Of the two principles mentioned (light-scattering and transmittance) the latter is more commonly used.

It is to be noted that in nephelometric determinations it is not the intensity of the light beam itself that is measured but that of the light scattered at right angles (see Fig. 140). Hence for low concentrations this method is said to be more sensitive, because a dark background represents zero concentration, whereas there is full illumination at zero concentration in turbidimetric measurements. The chemical principles for producing turbid systems are the same in both turbidimetry and nephelometry and the same precautions must be taken in their preparation and use.

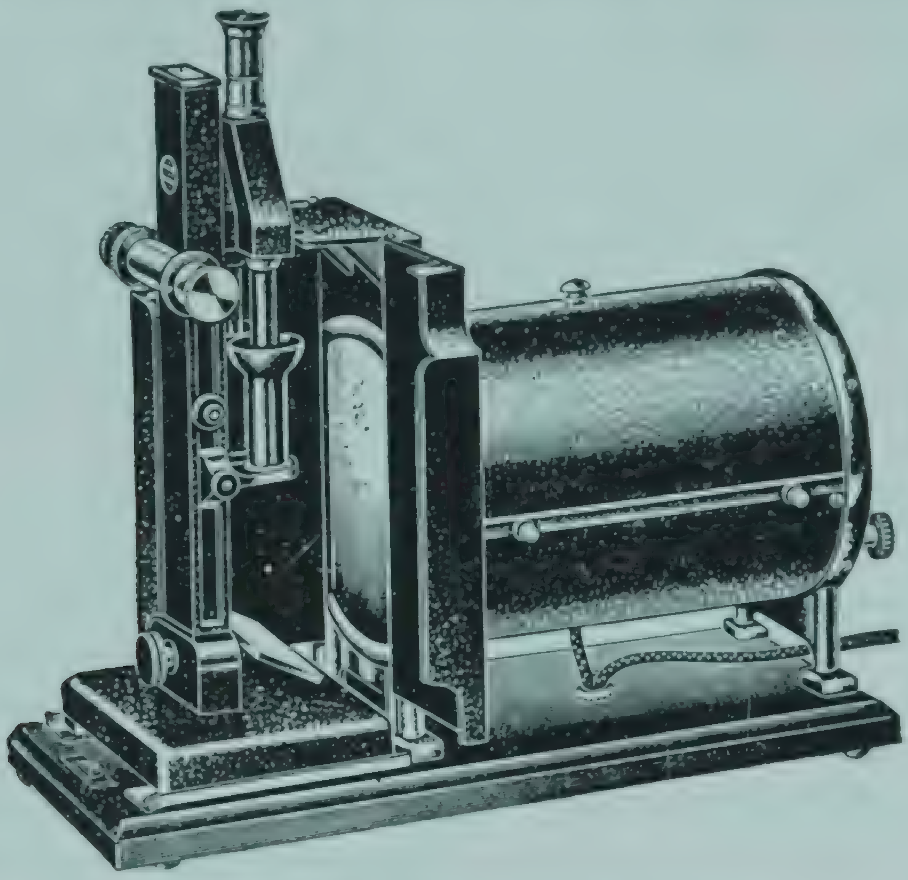

FIG. 140. KLETT NEPHELOMETER.

The instrument is especially constructed for nephelometric work, providing for parallel light which illuminates the cups at right angles. The cups are made of clear glass tubing with black bottoms.
Courtesy, Klett Manufacturing Co.

Turbidimetric measurements may be carried out by the same procedure and instruments used for the measurement of substances in solution, i.e., by comparison against a series of standards, by dilution, by varying the depth of solution, or by direct measurement of the light transmittance. Transmittance measurement, particularly when used in instruments equipped with photoelectric cells, is the most sensitive and satisfactory. The relationship between the amount of a substance in suspension and the turbidity or transmittance of the fluid is much more empirical than for substances in solution, depending as it does not only on the amount of material present but also on the size and shape of the suspended particles, their relative opacity or transparency, the relation between particle size and the wavelength of light used, and the uniformity with which a given turbidity may be reproduced. For a particular procedure, however, it may be found that over a limited range of concentration the turbidity or transmittance is directly proportional to concentration, and thus resembles optical density or extinction for substances in solution. In such an instance, turbidity measurement is carried out and results calculated in the same manner as for light absorption. In other cases, results must be obtained from a calibration curve constructed from known standards; it is even more important here than for substances in solution that the conditions prevailing at the time the standard turbidities were obtained be reproduced as closely as possible in an analysis.

If the substance in suspension is colorless in a colorless solvent, it may appear at first glance that the choice of wavelength or filter for photometric measurement is immaterial. This is not so, because of the influence of particle size on the scattering of light of different wavelengths ("Tyndall effect"). In general, light of shorter wavelength (at the blue end of the spectrum) is relatively more highly scattered than light of longer wavelength (red end of the spectrum), therefore the change in transmittancy for unit change in turbidity will be greater with short wavelengths than with long wavelengths. Thus the sensitivity of the procedure, or the relationship between scale readings and turbidity, may be considerably influenced by the wavelength employed. Other considerations may of course enter into the choice of wavelength; occasionally the interference of extraneous colored material in solution may be minimized by selection of a wavelength which is not absorbed by such material.

Turbidimetric measurements are employed not only for analytical purposes but also for the evaluation of particle size, and for determining the approximate number of plant or animal cells (yeast, bacteria, etc.) present in a fluid; this latter has had wide application, particularly in the field of microbiological assay. Turbidimetric estimation of red blood cell count has not as yet been successfully achieved. The use of turbidimetric methods in analytical chemistry has not been great, largely because of the difficulty of achieving a reproducible turbidity for a given concentration. Use has sometimes been made of a protective colloid to stabilize suspensions and promote uniformity; unfortunately, protective colloids usually distort the relationship between turbidity and concentration in such a way as to require a calibration curve which is highly empirical, and their use cannot be said to have solved the problems of turbidimetric analysis.

Fluorimetry. Certain substances are capable of absorbing light at one wavelength and radiating a portion of this absorbed light at some other wavelength. This phenomenon is known as fluorescence and may be used for analytical purposes, the intensity of fluorescence serving as a measure of concentration. Instruments designed for this purpose are known as fluorimeters. The fluorescence of the unknown may be compared visually against a standard or series of standards, or the intensity of the fluorescent light may be measured directly, using a light-sensitive device such as a photoelectric cell. Fluorimeters utilizing the latter principle are illustrated in Figs. 141—143.

The exciting light is usually in the ultraviolet portion of the spectrum, generally between 300 and 400 mμ, and the radiated light is usually in the visible region, but the applications of fluorescence are not limited to these conditions. Since the intensity of fluorescence is determined not only by those factors which affect light absorption (concentration, depth of solution, wavelength) but also by the intensity of the exciting light, measurements must be made at a uniform or controlled incident light intensity,

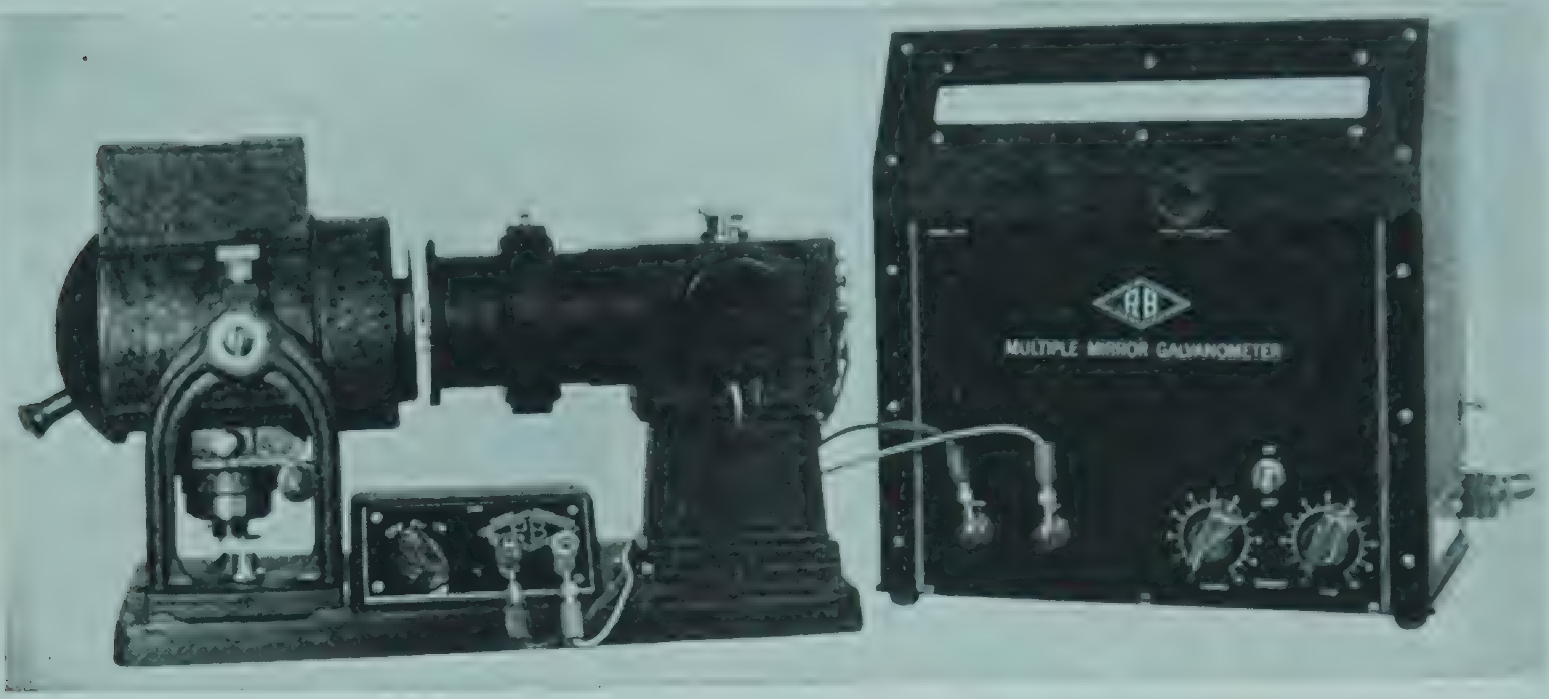

FIG. 141. FLUOROPHOTOMETER MODEL B.
Courtesy, Pfaltz & Bauer, Inc.

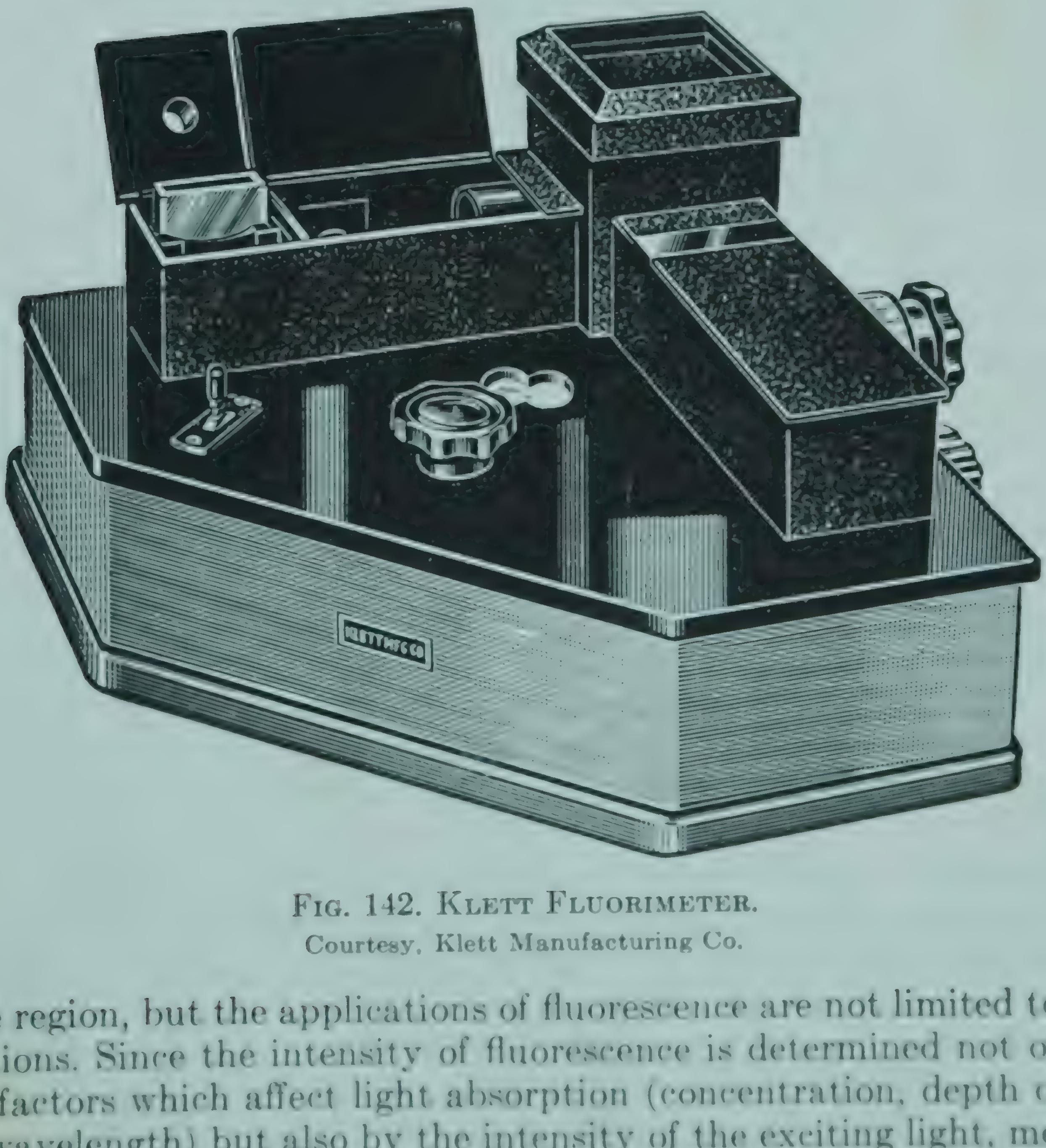

FIG. 142. KLETT FLUORIMETER.
Courtesy, Klett Manufacturing Co.

and the sample viewed or measured from the side of the light beam, to prevent interference from light transmitted through the solution. With photoelectric fluorimeters, results are usually obtained from a calibration curve relating the intensity of fluorescence to known concentrations of the substance; this relationship may be linear over a certain concentration range, in which case results can be obtained by calculation in terms of the fluorescence of a known standard. Permanent fluorescent standards such as uranium glass or solutions of quinine sulfate may also be used to control the calibration. Some substances show a "bleaching" or decrease in fluorescence during exposure to the exciting light; errors from this source may be minimized by rapid reading (short exposure), extrapolation, or the use of a low intensity of exciting light. Blank determinations to correct for intrinsic fluorescence of containers and reagents may be necessary; specificity may sometimes be enhanced by measuring the change in fluorescence after specific (e.g., enzymatic) destruction of the substance being determined. Quantitative analysis based upon fluorescence is limited at present to the determination of certain vitamins (see Chapter 35), drugs, dyes, and inorganic ions, but its usefulness should increase with further study.

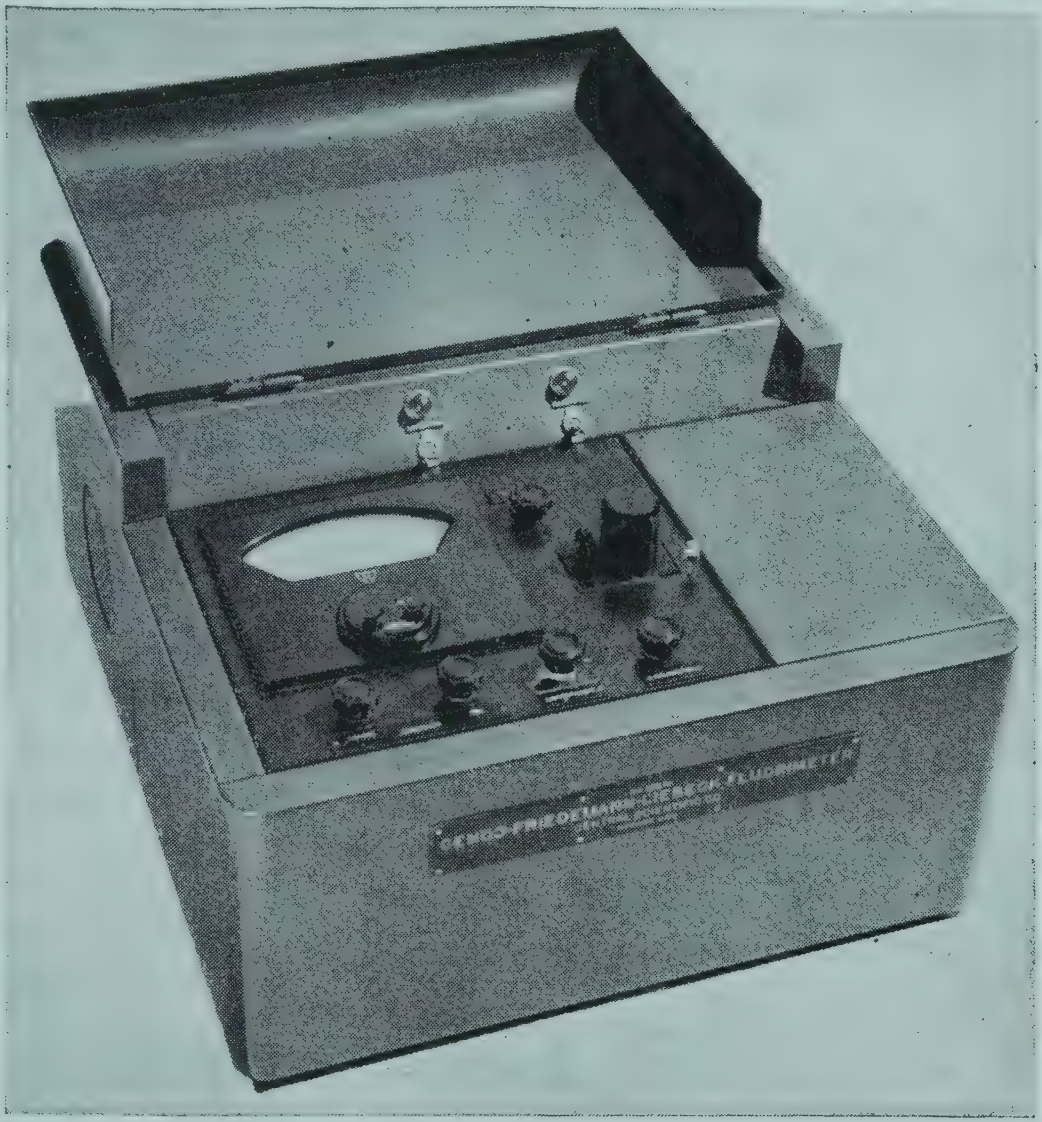

FIG. 143. CENCO-FRIEDEMANN-LIEBECK FLUORIMETER.
Courtesy, Central Scientific Co.

EXPERIMENTS ON PHOTOMETRY

1. Characteristics of Photometer. **Examine the photometer available and note the general principles of its design and operation. (This information** may be obtained from the instructor or from the manual usually supplied by the manufacturer of the instrument.) Is it a filter photometer or a spectrophotometer? Note particularly the type of scale used, and whether it is graduated in terms of per cent transmittancy, optical density, or units proportional to optical density. Place some distilled water in a photometer cuvette[11] and insert the cuvette in the instrument. Select any particular filter or wavelength setting, turn on the light, and adjust the photometer to its initial setting, which will represent either zero optical density or 100 per cent transmittancy—which is used? Now remove the cuvette from the light path. Does the adjustment change? Replace the cuvette. Does the reading return to its initial value? If it does not, allow the light to burn for about 5 minutes, reset to zero (or 100) and repeat the test of stability. Is there any increase in stability after the light has been on a short while? Why is it necessary for photometer readings to be based on a constant initial setting?

2. *Beer's Law.* Obtain some defibrinated or oxalated blood. Dilute 1 ml. of this to 100 ml. in a volumetric flask with dilute ammonia solution (4 ml. of concentrated ammonium hydroxide per liter of water). Mix well by inversion. Prepare a series of known dilutions of this "stock standard" as follows: into test tubes place 0.0, 1.0, 2.0, 3.0, etc., up to 10.0 ml. portions of the stock solution, and add sufficient dilute ammonia solution to each tube to make the final volume 10 ml. in each case. Mix. Using light of 520 mμ wavelength, adjust the photometer to zero optical density (or 100 per cent transmittance) with the contents of the first tube, which is a "reagent blank." Determine the photometer readings for each of the other solutions, checking the initial setting with the blank solution once or twice during the series of readings; if any change has occurred reset the photometer before continuing with the readings.

On a sheet of cross-section paper, plot the photometer readings on the y-axis against equivalent concentration (0, 1, 2, 3, etc., up to 10) on the x-axis, and connect the points by a smooth curve. Which of the four types of curves shown in Fig. 127, p. 517, do you obtain? What is the relation between photometer readings and concentration? If the photometer scale reads in terms of per cent transmittancy, (*a*) compute the value of log T_s for each concentration and plot this value against concentration on a second sheet of cross-section paper (or plot T_s on the logarithmic axis of semilogarithmic paper, against concentration on the linear axis), and (*b*) convert transmittancy into optical density by the use of the table on p. 520, and plot optical density against concentration, using ordinary cross-section paper. What types of curves should be obtained if Beer's law is valid? Do your results indicate that hemoglobin solutions obey Beer's law under the conditions you are using? How may your results be used as a basis for the photometric determination of hemoglobin?

Assume that one of your solutions is a standard, of known concentration, and that the others are unknowns. Calculate their concentrations relative to that of the standard, from the determined optical densities, as described on p. 518. Is Beer's law applicable over the entire range of concentration you are

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[11] Cuvettes, cells, and test tubes used in photometric instruments must be thoroughly clean and free of scratches. A chromic-sulfuric acid solution may be employed, followed by thorough rinsing with distilled water. Do not use alkaline detergent solutions, because of the danger of etching the polished surfaces. Use a fresh piece of soft lintless paper, such as lens paper or Kleenex, to wipe dry the outside of the vessel before placing it in the instrument. Care should be taken not to let the fingers touch the glass surfaces which will be exposed to the light path. A handkerchief or towel should not be used for wiping cuvettes, etc.

using? What must be done in a photometric analysis if (*a*) the procedure does not obey Beer's law, or (*b*) a reading falls outside the range of application of Beer's law?

3. *Relation between Transmittancy and Wavelength.* Repeat Exp. 2, but use a wavelength in (*a*) the blue portion of the spectrum, and (*b*) the red portion. Plot the results graphically for each wavelength used, and compare with the results of Exp. 2. How may the wavelength chosen influence the sensitivity of a photometric procedure? What is the indication on your graphs of the sensitivity of this particular procedure? Suppose hemoglobin were a contaminant of a solution, instead of the substance being measured; at what wavelength would its presence cause the least error?

4. *Relation between Transmittancy and Depth of Solution.* Repeat Exp. 2, but make measurements at some other depth of solution, if such facilities are available. If not, compute the theoretical reading for each concentration at a depth of solution twice that used in Exp. 2. Plot the data graphically and compare with the previous results. What is the effect of solution depth on the transmittancy value for a given concentration? How may this be used to increase the sensitivity of a photometric procedure?

5. *Correcting for a Blank Color.* Set up the aliquots of 1:100 hemoglobin stock solution used in Exp. 2 (up to 9 ml.) but before diluting to 10 ml. with dilute ammonia solution, add to each tube 0.5 ml. of a dilute ammonia solution to which a few drops of blood have been added, sufficient to give a noticeable pink color. Thus each solution now contains a relatively known amount of hemoglobin, plus a constant blank color. Adjust the photometer to its initial setting with the blank tube, and read the other solutions relative to this, as before. Plot the data as described for Exp. 2. Does the presence of a constant blank color influence the photometric calibration, if the photometer is set to its initial reading with the blank solution? Since optical density is additive—i.e., the total optical density of a solution is the sum of the densities due to the various light-absorbing substances present—what other way can you suggest of correcting for the density of a blank?

6. *Effect of Time of Standing on Transmittancy.* Obtain some defibrinated blood, and dilute 0.1 ml. to 25 ml. with 0.1 N hydrochloric acid. Mix by inversion, transfer a portion to a photometer cuvette, and read immediately, the photometer having been previously set to zero optical density at 520 mμ with the dilute acid alone. Repeat the reading on this same sample at suitable time intervals, say every five minutes, for one hour or until the reading becomes constant. Be sure to check the initial setting of the photometer before each reading. Plot the relationship between time of standing and photometer reading. Many of the colors produced in common photometric procedures show a similar behavior with respect to time of standing. What does this experiment show concerning the desirability of time control in photometric analysis?

7. *Calibration of Test Tubes Used as Cuvettes.* Many types of photometers are designed to use interchangeable test tubes of uniform dimensions as solution containers or cuvettes. If the tubes are truly interchangeable, they should all give the same reading for a given colored solution, and they may be tested on this basis. Prepare a stable colored solution of such intensity that it shows by trial a reading about in the middle of the photometer scale.

A dilute solution of hemoglobin, as described in previous experiments, may be used; an equally satisfactory test solution may be made by diluting ordinary India ink with water to a suitable intensity. Place portions of this test solution in each of the test tubes, and read them in the photometer, using water for the initial setting and checking this setting at suitable intervals. Select all those tubes which give identical or nearly identical readings, and discard the remainder. Sometimes two lots of tubes may be obtained, the readings for each lot centering with the desired accuracy around a particular reading, but a different reading in each case. Each lot may then be used independently, but they should be marked so that the lots will not be mixed. It is also well to mark the tubes so that they will always be placed in the photometer in the same position. Test tubes calibrated in this way are as accurate for photometric purposes as rectangular cuvettes, and much more convenient. A tube should be discarded when it becomes scratched, or when checking shows it to have lost its interchangeability.

GENERAL PROCEDURES IN BLOOD ANALYSIS

Drawing Blood for Analysis. Except when analytical methods permit the use of a few drops of blood (drawn from the finger tip, toe, or ear lobe), samples of blood are usually obtained by venipuncture.

Procedure. Draw a tourniquet (of soft, firm rubber tubing or a strip of bandage) tightly about the arm of the patient a couple of inches above the elbow. Have the patient clench his fist firmly. Wash the skin surface about the most prominent vein on the inner surface of the elbow (usually the median basilic) with 70 per cent alcohol, allow to dry, hold the vein immobile with the thumb, and into the vein insert a sharp, sterile hypodermic needle (No. 18, an inch and a half long) which is attached to a dry sterile syringe of suitable capacity. The needle should penetrate the vein from the side and at an angle of about 50° with the surface of the arm, the bevel or opening of the needle being kept upward or to the side. As soon as blood is seen to enter the syringe, retract the plunger slowly until the desired amount of blood has entered the syringe. Before removing the needle from the vein, loosen the tourniquet, have the patient unclench his fist, and on the skin at the point of entrance of the needle hold in place a small pad of folded gauze moistened with 70 per cent alcohol. Withdraw the needle, detach it from the syringe, and into a suitable container eject the blood from the syringe (not too vigorously, which might cause hemolysis). Pressure on the gauze pad for a few minutes will effectively prevent bleeding from the skin puncture.

The use of a syringe is not essential; the needle alone may be used. The blood is allowed to flow from the free end of the needle into a suitable container until the desired amount has been obtained. The needle is then withdrawn as described above. For special precautions to be used in drawing blood where the maintenance of physiological gas tensions is important, as in the determination of carbon dioxide content, etc., see Chapter 24.

Preparation of Whole Blood and of Plasma. When whole blood or plasma is desired for analysis, the blood must be treated with anticoagulant before clotting commences. The most convenient way to do this is to have containers for the blood already prepared with sufficient anticoagulant in the form of a thin dried film over the inside surface (see below). The thin film promotes quick solubility and mixing with the added blood. Test tubes or small wide-mouth bottles of approximately 1-ounce capacity may be used as containers.

Procedure. **Transfer the blood, as quickly as possible after drawing, from the syringe to a container which has sufficient anticoagulant in the form of a thin dried film to prevent clotting of the blood. Mix by rotation gently but thoroughly to dissolve and distribute the anticoagulant. The blood is now ready for use. To obtain plasma, centrifuge and remove the supernatant plasma with a rubber-bulb pipet.**

Blood specimens are best taken in the morning before breakfast, to minimize the influence of food ingestion. Analyses are preferably made as soon as possible after the blood is drawn; during any interval between drawing and analysis, the blood should be kept cold and well stoppered to minimize evaporation. For blood sugar analyses in particular, the protein-free filtrate should be prepared as soon as possible, to minimize loss of sugar by glycolysis.[12] Protein-free filtrates will keep better than whole blood or plasma, particularly if kept cold and in the presence of a drop of toluene as preservative.

Anticoagulants. The most commonly used anticoagulant is neutral potassium oxalate, of which from 1 to 2 mg. are required per ml. of blood. Only an amount of anticoagulant sufficient for the quantity of blood to be received should be employed; excessive amounts of anticoagulant may interfere with some analyses, cause hemolysis, or produce an abnormal distribution of water and electrolytes between cells and plasma.

Procedure. **To prepare containers with sufficient potassium oxalate for 6 to 10 ml. of blood, prepare a stock solution of 10 per cent neutral potassium oxalate and pipet 0.1 ml. of this into each container. Rotate to produce maximal spreading; then place in an incubator (or oven at 100° C.) to dry. The oxalate should form a thin dry film on the sides of the container. For smaller quantities of blood, use half as much stock solution. It is good practice to have two sets of containers, suitably labeled, one for 3 to 5 ml. of blood, and one for 6 to 10 ml. of blood.**

Other anticoagulants, and the amounts required per ml. of blood, include sodium citrate (5 mg.), lithium or sodium oxalate (1 to 2 mg.), sodium fluoride (10 mg.), and heparin (0.2 mg.). Containers may be prepared with the proper amounts of these anticoagulants in a manner similar to that described above for potassium oxalate. Of the various anticoagulants, heparin is by far the most satisfactory and should be more widely used. Sodium fluoride acts as a preservative[13] and has the advantage of inhibiting glycolytic decomposition of blood sugar, but interferes with certain methods. A special mixture (Heller and Paul)[14] of ammonium oxalate (3 parts) and potassium oxalate (2 parts) has the advantage of causing no change in red cell volume, and hence is useful for hematocrit determinations and for methods involving the measurement of specific gravity of whole blood or plasma. It cannot be used in ordinary blood analytical procedures because of the presence of ammonia.

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[12] Denis (*J. Biol. Chem.*, **44**, 203 (1920)) has shown that for the Folin-Wu sugar method, blood may be preserved for four days or more at room temperatures if 1 drop of commercial formalin (40 per cent) solution is added to each 5 ml. of blood.

[13] John (*Arch. Path. Lab. Med.*, **1**, 227 (1926) after an extensive study of blood preservatives, recommends sodium fluoride and thymol in the proportion of 20:1, using 20 mg. of the mixture per 10 ml. of blood. Roe, Irish, and Boyd (*J. Biol. Chem.*, **75**, 685 (1927)) find 10 mg. NaF per ml. blood preserves for 10 days as far as nonprotein nitrogen, uric acid, creatinine, sugar, and cholesterol are concerned, provided the blood is sterile. If not, at least 20 mg. are

Preparation of Blood Serum for Analysis. In the collection of blood for serum, care must be taken to avoid trauma and hemolysis.

Procedure. **When serum rather than whole blood or plasma is desired for analysis, place the freshly drawn blood directly into a small test tube without anticoagulant. Allow to clot at room temperature and then chill thoroughly in the refrigerator. Centrifuge down the clot and remove the supernatant serum with a rubber-bulb pipet.**

If a centrifuge is not available, the blood may be allowed to clot with the tube in a slanting position; then after chilling overnight in an upright position the serum may be poured off from the side of the tube opposite the slanting clot.

Measurement of Blood. Because of its physical characteristics and the presence of suspended red cells, whole blood is much more difficult to measure exactly than ordinary fluids. Serious errors have been traced to faulty measurement of blood. Before measurement, the sample must be thoroughly mixed to ensure uniform distribution of cells and plasma.

Procedure. **To mix whole blood without trapping air bubbles, if the sample is in a test tube use a small footed stirring rod which is raised and lowered in the sample a sufficient number of times to ensure complete mixing. If the sample is in a wide flat bottle, place the bottle firmly on the table top and vigorously trace a 1-foot circle with the bottle flat against the table top, for at least a dozen times or until the blood is uniformly mixed. Measure out the portion of blood immediately after mixing, and repeat the mixing procedure before each new measurement.**

To measure blood accurately, draw the blood up into a transfer pipet until the level is a little above the graduation mark. Wipe off excess blood from the pipet tip, allow to drain slowly until the blood level is exactly at the mark, and again wipe off excess blood. Deliver the blood into the receiver slowly, adjusting the rate of delivery by finger pressure on the mouthpiece of the pipet so that as the blood drains from the pipet, the pipet walls remain clear

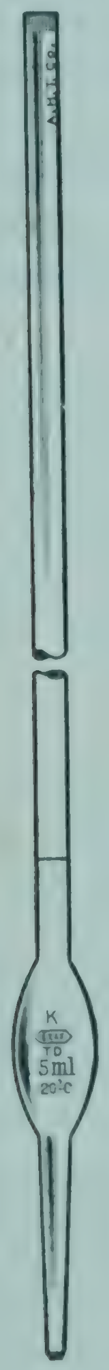

FIG. 144. OSTWALD-FOLIN PIPET.

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required, but even this gives poorer results than are obtained with sterile blood. Interference of NaF with urease action is overcome by dilution with 7 to 10 volumes of water if not over 30 mg. of NaF per ml. are used.

[14] Heller and Paul: *J. Lab. Clin. Med.*, 19, 777 (1934).

and there is no visible film of blood remaining behind. When the blood has drained completely, blow out the last drop into the receiver.[15]

Ordinary pipets may be used for blood measurement, with slight error due to the fact that they are usually calibrated for the delivery of water rather than blood. Pipets calibrated "to contain" ("TC"), such as are commonly used in microanalyses, are not subject to this error, since they are designed for delivery of the blood into a second fluid, the resulting mixture then being used to rinse out any blood remaining in the pipet. Folin proposed the use of the Ostwald type pipet (Fig. 144), which has less surface per unit volume than the ordinary pipet, and no sharp shoulders to impede drainage, and these have found favor in many laboratories. Where the amount of blood available is limited and it is desired to use as much as possible, a pipet graduated to the tip is useful (Fig. 145). In using this type of pipet, the greatest error is at the tip, particularly when the tip is imperfect, and if possible the measurement should exclude this portion (i.e., for a 5-ml. portion, measure from the 6-ml. mark to the 1-ml. mark). In general, the most accurate method of measurement is by careful drainage between two accurately established graduation marks. Volumetric pipets constructed on this principle are not generally available; ordinary graduated pipets should be used in this manner whenever possible.

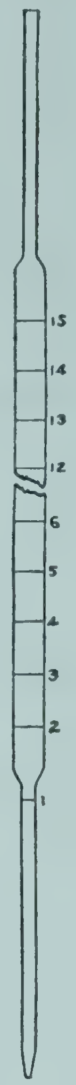

FIG. 145. DILUTING PIPET.

Courtesy, Folin and Wu: *J. Biol. Chem.*, **38,** 81 (1919).

PREPARATION OF THE PROTEIN-FREE BLOOD FILTRATE

Method of Folin and Wu.[16] **Principle.** The proteins of whole blood, plasma, or serum are removed by precipitation with tungstic acid (formed by the interaction of sodium tungstate and sulfuric acid) and filtration. The filtrate is suitable for the determination of the following: nonprotein nitrogen, urea, uric acid, creatine and creatinine, sugar, amino acids, and chlorides. Sufficient filtrate for one or two determinations is provided by 2 ml. of blood; for all the determinations, about 10 ml. of blood are needed.

Procedure.[17] **Transfer a measured quantity of blood to a flask having a capacity at least 15 times that of the volume taken. For each volume (*A* ml.) of blood taken, add from a buret exactly 7 volumes (7 × *A* ml.) of water and**

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[15] This is for transfer pipets which are calibrated for blowout delivery. Such pipets are commonly marked by the manufacturer with an etched ring around the pipet near or at the top. If the pipet is calibrated for drainage delivery, allow to drain for 1 minute with the tip of the pipet touching the wall of the receiver. For graduated pipets, allow the blood to run out either to the desired graduation mark, or to the tip if the pipet is graduated to the tip.

[16] Folin and Wu: *J. Biol. Chem.*, **38,** 81 (1919).

[17] Reagents Required: *Sodium tungstate, 10 Per Cent Solution.* Dissolve 100 g. of reagent-

mix. Add 1 volume (*A* ml.) of 10 per cent sodium tungstate solution, and mix. Finally add slowly and with shaking 1 volume (*A* ml.) of two-thirds normal sulfuric acid. Stopper the flask and shake it. Only a few bubbles should form as a result of this shaking if all the proteins have been precipitated. Let stand for 10 minutes. The color of the mixture should change from red to dark brown. If this change in color does not occur, the coagulation is incomplete, usually because too much oxalate is present. In such an emergency the sample may be saved by adding 10 per cent sulfuric acid, drop by drop with shaking, until there is no foaming and until the dark brown color has set in.

Pour the mixture on a dry folded filter large enough to hold it all. Cover the funnel with a watch glass to minimize evaporation. Collect the filtrate in a clean dry container. If the first few drops of filtrate are not absolutely clear, return this portion to the funnel and replace the receiver with a fresh one. Allow to filter until as much filtrate as possible has been obtained.

For plasma or serum the procedure is similar except that 8 volumes of water and only ½ volume of both tungstate and acid are used.

The following modifications of the Folin-Wu precipitation employ fewer solutions and yield more filtrate.

Haden's*[18] *Modification. **Add directly to the blood from a buret, 8 volumes of N/12 sulfuric acid.[19] Laking and darkening occur rapidly. Add 1 volume of 10 per cent sodium tungstate, shake well, and filter, as above.**

Van Slyke and Hawkins'*[20] *Modification. **Add directly to the blood, 9 volumes of a mixture of 8 parts N/12 sulfuric acid and one part 10 per cent sodium tungstate. Shake and filter, as above. The precipitating solution should be clear and not more than two weeks old.**

Blood filtrates prepared by any of the above methods represent a 1:10 dilution of the sample; that is, 1 ml. of filtrate corresponds to 0.1 ml. of original material. Since any error in measurement of the added reagents will produce a corresponding error in calculations, which are based upon the assumption of an exact 1:10 dilution, all measurements must be made carefully, preferably by the use of calibrated pipets or burets.

The protein-free filtrates are not acid enough to prevent bacterial decomposition. If the filtrates are to be kept more than about 12 hours a few drops of toluene or xylene should be added. For optimum precipitation of protein, the filtrate should not be more alkaline than pH 2.8.[21] A

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grade, carbonate-free sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) in water and dilute to 1 liter. Stable indefinitely.

Two-thirds Normal Sulfuric Acid. Weigh out 35 g. of concentrated sulfuric acid in a small tared beaker, dilute to 1 liter with water, and mix. Check by titration against standard alkali and adjust if necessary. The two-thirds normal acid is intended to be equivalent to the sodium tungstate, so that when equal volumes are mixed substantially the whole of the tungstic acid is set free without the presence of an excess of sulfuric acid. The liberated tungstic acid is taken up almost quantitatively by the blood proteins, to yield a filtrate which is only slightly acid.

[18] Haden: *J. Biol. Chem.*, **56**, 469 (1923).

[19] Equivalent to combining 1 volume of ⅔ N acid with 7 volumes of water. Add 2.5 ml. of concentrated sulfuric acid to 1 liter of distilled water. Mix well and check by titration against 0.1 N NaOH. 20 ml. of N/12 acid should require 16.7 ml. of 0.1 N NaOH for its neutralization.

[20] Van Slyke and Hawkins: *J. Biol. Chem.*, **79**, 739 (1928).

[21] Merrill: *J. Bioi. Chem.*, **60**, 257 (1924).

drop of 0.04 per cent bromophenol blue added to a few drops of filtrate on a test plate should give a yellow or greenish-yellow color, alkalinity being denoted by a pure blue shade.

Other Methods of Deproteinization. Although tungstic acid filtrates prepared as described above are the most satisfactory for general purposes, many other reagents have been used for the deproteinization of blood and other biological fluids. Examples of the use of certain of these (zinc and copper hydroxides, trichloroacetic acid, etc.) will be found subsequently in this chapter. In most cases they are employed in particular analytical techniques in which tungstic acid filtrates have been found unsatisfactory for one reason or another. Hiller and Van Slyke[22] have reported on the comparative value of a number of common protein precipitants. Benedict and Newton[23] recommend the use of tungstomolybdic acid in place of tungstic acid for the preparation of blood filtrates, not only for general purposes but also specifically for the determination of blood ergothioneine, for which tungstic acid filtrates are unsatisfactory.

DETERMINATION OF NONPROTEIN NITROGEN

Introduction. The nonprotein nitrogen (NPN) of the blood is a collective concept and includes the nitrogen from all of the nonprotein nitrogenous constituents of blood which are found in a protein-free filtrate, such as urea, uric acid, creatine and creatinine, amino acids, glutathione, and many others in small amount, some of which are of unknown nature. Of these various substances, the compound urea contributes by far the largest share to the total, urea nitrogen representing ordinarily about 45 per cent of the blood NPN. The nonprotein nitrogen content of blood is usually determined by various micro modifications of the standard Kjeldahl method for the determination of total nitrogen (see Chapter 31).

1. *Method of Folin and Wu:*[24] *Principle.* Nitrogen is determined in a portion of the protein-free blood filtrate by a micro-Kjeldahl method, using a sulfuric and phosphoric acid mixture for the digestion, the ammonia formed being determined colorimetrically after direct nesslerization of the digestion mixture.

***Procedure.*[25] Transfer 5 ml. of blood filtrate to a large test tube (pyrex) 200 mm. × 25 mm., graduated at 35 ml. and 50 ml. The test tube should either be dry or rinsed with alcohol to reduce the danger of bumping. Add 1 ml. of diluted acid mixture and a quartz pebble. Boil vigorously over a**

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[22] Hiller and Van Slyke: *J. Biol. Chem.*, **53**, 253 (1922).

[23] Benedict and Newton: *J. Biol. Chem.*, **83**, 357 (1929).

[24] Folin and Wu: *J. Biol. Chem.*, **38**, 81 (1919). For a gasometric method, see Van Slyke: *J. Biol. Chem.*, **71**, 235 (1927).

[25] Reagents Required: *Diluted Acid Mixture.* Made by diluting "regular acid mixture" with an equal volume of water. To prepare "regular acid mixture," add 300 ml. of 85 per cent phosphoric acid, reagent grade, to 50 ml. of a 5 per cent copper sulfate solution. Add 100 ml. of reagent-grade concentrated sulfuric acid and mix. Keep well stoppered to prevent absorption of ammonia from the air.

Nessler's Solution. See Appendix.

Standard Ammonium Sulfate Solution. Dissolve exactly 0.236 g. of reagent-grade ammonium sulfate in water, transfer quantitatively to a 1-liter volumetric flask with rinsings, add a few drops of concentrated sulfuric acid, make up to volume with water, and mix. This microburner until the characteristic dense fumes begin to fill the tube. This will happen in from 3 to 7 minutes, depending on the size of the flame. When the test tube is nearly full of fumes reduce the flame sharply so that the speed of the boiling is reduced almost to the vanishing point. Cover the mouth of the test tube with a watch glass. Continue the gentle heating for 2 minutes, counting from the time the test tube became filled with fumes. If the oxidation is not visibly finished at the end of 2 minutes the heating must be continued until the solution is nearly colorless. At the end of 2 minutes remove the flame and allow the digestion mixture to cool for 70 to 90 seconds. Then add 15 to 25 ml. of water. Cool further approximately to room temperature and then fill with water to about 1 cm. under the 35-ml. mark. Set aside until the standard is ready, since in this determination it is important that color development be carried out in both unknown and standard under as nearly identical conditions as possible.

A standard suitable for either colorimetric or photometric measurement is prepared as follows: transfer 3 ml. of a standard solution of ammonium sulfate, containing 0.15 mg. of nitrogen, to a graduated tube similar to that used for the blood filtrate, add 1 ml. of the diluted phosphoric-sulfuric acid mixture, to balance the acidity of the unknown, and dilute with water to about 1 cm. under the 35-ml. mark. For photometric measurement a third or blank tube is required, containing 1 ml. of diluted acid mixture alone, made up with water as described for unknown and standard.

When all the tubes are ready, nesslerize each individually as follows: measure out 15 ml. of Nessler's solution in a graduated cylinder, set the tube contents in motion by gentle rotatory shaking, and in one continuous motion pour the Nessler solution into the tube. Do not wait for the Nessler solution to drain completely from the cylinder, since the exact amount of Nessler solution is relatively unimportant, but immediately add water to the tube contents up to the 50-ml. mark, insert a clean rubber stopper, and mix by inversion. Proceed immediately to the nesslerization of the remaining solutions in a similar manner. Allow the solutions to stand 10 minutes after adding the Nessler reagent and mixing, to permit maximum color development, and read within the next 10 minutes or so. Prolonged standing may lead to the development of a turbidity which renders the color comparison difficult or even worthless.[26] Occasionally a turbidity is present before nesslerization, due to the action of the acid digestion mixture on the glass of the tube. This turbidity can be removed by centrifuging a portion of the colored solution before color comparison.

For colorimetric measurement, match the standard against itself at 20 mm. in the usual way, and then compare the unknowns against the standard. For photometric measurement, transfer portions of the colored solutions to suitable containers and determine the densities in the pho-

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solution is stable indefinitely and contains 0.05 mg. of nitrogen per ml. Dry the ammonium sulfate before weighing it out by heating in an oven overnight at 100° C.

In all nitrogen analyses involving the determination of small amounts of ammonia, only the highest purity ammonia-free reagents may be used, and solutions must be kept protected to prevent the absorption of ammonia from the air. Distilled water containing ammonia cannot be used; to free it from ammonia, redistil in the presence of a little sulfuric acid in an all-glass still, and protect the distillate from exposure to ordinary laboratory air by a trap containing dilute sulfuric acid.

[26] Chilling all solutions in cold water for 10 minutes prior to adding the Nessler reagent has been recommended to prevent the formation of turbidity before reading, as has also the addition of a few drops of 2 per cent gum ghatti solution (see Appendix) to standard and unknowns just before nesslerization. The use of gum ghatti solution is not recommended unless absolutely necessary; for further discussion, see footnote 35, p. 551.

tometer at 480 to 540 mμ (see below), setting the photometer to zero density (100 per cent transmittance) with the blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.15 \times \frac{100}{0.5} = \text{mg. of NPN per 100 ml. of blood}$$

The standard indicated is satisfactory for the range of 15 to 60 mg. per cent blood NPN. For amounts outside this range, repeat the analysis using less or more filtrate as necessary, and correct the calculations accordingly.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.15 \times \frac{100}{0.5} = \text{mg. of NPN per 100 ml. of blood}$$

The spectrophotometric characteristics of the Nessler reaction with ammonia are illustrated in Fig. 240, p. 879. For the amounts of nitrogen which are concerned here, satisfactory agreement with Beer's law is found with any filter or wavelength setting between 480 and 540 mμ, the choice depending largely upon the sensitivity desired. In a 1-cm. cuvette, at 480 mμ, the density of the standard prepared as described above is approximately 0.250. This means that up to about 120 mg. per cent blood nonprotein nitrogen may be accurately determined under these conditions. For higher values, or with deeper cuvettes, where the range is proportionately reduced, use less filtrate for the analysis and correct the calculations accordingly.

Interpretation.[27] The nonprotein nitrogen content of normal blood ranges from 25 to 35 mg. per 100 ml. Variations in blood NPN content are ordinarily due largely to variations in urea content. Increased values for nonprotein nitrogen are observed in nephritis, more especially in the chronic types and in terminal stages, where they may have prognostic significance, although uremic symptoms may develop without marked elevation of nonprotein nitrogen. The direction of change will impart greater information than the magnitude of an isolated determination.

In regard to the choice of determining either the nonprotein or the urea N as a clinical test of renal function, the advantages seem to be in favor of the latter, for the following reasons: urea covers a relatively wider range of variation in disease; it is a single chemical compound rather than a mixture of partly undetermined composition; it is simple to determine clinically, especially if direct nesslerization of the Folin-Wu filtrate is employed. It is of interest, however, that the urea fraction of the nonprotein nitrogen, which usually rises in renal disease, shows subnormal values in cases of eclampsia, suggesting an increase in the "rest N" fraction which may contain toxic split-products of protein metabolism.

2. Method of Koch and McMeekin:[28] ***Principle.*** This method differs from the preceding one in that sulfuric acid and hydrogen peroxide are used in the digestion.

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[27] For review, see Folin: *Physiol. Revs.*, **2,** 460 (1922); also Peters and Van Slyke's book (see Bibliography at end of chapter).

[28] Koch and McMeekin: *J. Am. Chem. Soc.*, **46,** 2066 (1924). A stronger acid digestion mixture containing perchloric acid is used by Rose: *J. Biol. Chem.*, **64,** 253 (1924). A saturated solution of NH_3-free potassium persulfate is recommended by Wong: *J. Biol. Chem.*, **55,** 431 (1923). There is some evidence that both hydrogen peroxide and perchloric acid may yield inconstant results, possibly through oxidation of a portion of the ammonia, although the procedures appear to be sufficiently reliable for routine clinical purposes. Selenium is said to be satisfactory as an oxidation catalyst, and appears to give good results (see Campbell and Hanna: *J. Biol. Chem.*, **119,** 1 (1937)).

Procedure.[29] Transfer 5 ml. of the 1:10 protein-free filtrate to a 200 × 25 mm. pyrex test tube, add 1 ml. of 1:1 sulfuric acid and a small quartz pebble, and heat over a microburner to evaporate off the water. When charring begins and white fumes begin to appear in the tube, reduce the size of the flame or raise the tube so that the tip of the flame just touches the bottom of the tube. Continue heating until no further darkening occurs. Remove the flame, allow the tube contents to cool for about 1 minute, and then add 1 drop of 30 per cent hydrogen peroxide, allowing it to drop directly into the solution. Replace the flame and heat again to boiling. If the solution is not decolorized repeat the addition of the hydrogen peroxide. Finally boil gently for 5 minutes. Cool, transfer to a 50-ml. volumetric flask with about 35 ml. of water, and set aside until the standard is ready. Test tubes graduated at 35 and 50 ml. as described for the previous procedure may be used here instead of volumetric flasks. To prepare the standard, transfer 3 ml. of standard ammonium sulfate solution, containing 0.15 mg. of nitrogen, to a 50-ml. volumetric flask, add 1 ml. of 1:1 sulfuric acid, and dilute to about 35 ml. with water. For photometric measurement a blank is prepared in a third flask by diluting 1 ml. of 1:1 sulfuric acid to about 35 ml. with water.

When ready, to each flask add 12 ml. of modified Nessler reagent from a graduated cylinder, swirling the contents of the flask just before adding the Nessler reagent to promote quick and uniform mixing. Dilute immediately with water to the 50-ml. mark, stopper, and mix by inversion. Allow to stand 10 minutes before reading. Read in the colorimeter or photometer exactly as described for the previous method, using the same calculations.

Interpretation. See previous method.

Other Methods. As has been indicated, most of the modifications in the micro-Kjeldahl determination of blood nonprotein nitrogen by direct nesslerization involve changes in the digestion mixture. Alternate procedures for the determination of the ammonia formed include gasometric estimation (Van Slyke, *loc. cit.*); aeration of the ammonia from the alkalinized digest as described for the determination of urea (see next section); or steam distillation of the ammonia (see Chapter 31). In both aeration and steam distillation, the recovered ammonia is absorbed in acid and may be estimated by titration or by nesslerization; the advantage of nesslerization after separation of the ammonia from interfering material is that crystal-clear colored solutions are invariably obtained. A steam distillation device such as that illustrated in Fig. 241, p. 881, or its equivalent, is convenient, almost automatic in operation, and requires but a few minutes for each sample. Steam distillation prior to estimation of ammonia is recommended for all precise micro-Kjeldahl analyses. For very small amounts of nitrogen (ammonia), the microdiffusion method of Conway (see pp. 668 and 886) has given excellent results.

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[29] Reagents Required: *Sulfuric Acid*, 1:1. With stirring, carefully add 50 ml. of concentrated sulfuric acid to 50 ml. of water. Cool and keep well stoppered to prevent absorption of ammonia from the air.

30 Per Cent Hydrogen Peroxide. Only the highest grade, low-nitrogen reagent (Merck's or Baker's are satisfactory) may be used. This reagent is extremely corrosive to the skin and must be dispensed with care, preferably by the use of a rubber-bulb pipet. Keep in the refrigerator when not in use.

Modified Nessler Solution. See Appendix.

Standard Ammonium Sulfate Solution. See previous method.

DETERMINATION OF UREA

Introduction. Practically all of the methods in use at the present time for the determination of the urea content of blood are based upon incubation with preparations of the enzyme urease,[30] whereby urea present is converted into ammonium carbonate. The ammonia formed may then be determined directly by colorimetric methods, or separated by either aeration or distillation and then determined either colorimetrically or titrimetrically. The carbon dioxide produced by decomposition of ammonium carbonate may also be measured gasometrically.[31] Several methods which do not involve the use of urease have also been described.[32] The choice of procedure among the many available appears to be largely a question of the facilities and requirements of the individual laboratory.

1. Method of Folin and Svedberg:[33] ***Principle.*** The ammonia produced by the action of urease on the protein-free blood filtrate is distilled off and determined colorimetrically by reaction with Nessler's reagent. For a discussion of the accuracy of this method see Gentzkow (*J. Biol. Chem.*, **143,** 540 (1942)).

Procedure.[34] **Transfer 5 ml. of tungstic acid blood filtrate to a pyrex test tube of 30 ml. capacity (tubes which previously contained Nessler solution should be rinsed with concentrated nitric acid and then with water before use).**

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[30] Marshall: *J. Biol. Chem.*, **15,** 487 (1913).

[31] Van Slyke: *J. Biol. Chem.*, **73,** 695 (1927).

[32] Ormsby: *J. Biol. Chem.*, **146,** 595 (1942); Barker: *ibid.*, **152,** 453 (1944); Archibald: *ibid.*, **157,** 507 (1945). See also method of Leiboff and Kahn (p. 555).

[33] Folin and Svedberg: *J. Biol. Chem.*, **88,** 77 (1930).

[34] Reagents Required: *Acetate Buffer Solution.* Dissolve 15 g. of crystallized sodium acetate in a 100 ml. volumetric flask by the help of 50 to 75 ml. of water. Add 1 ml. of glacial acetic acid, dilute to volume, and mix.

Urease Solution. Transfer 0.5 g. of jack bean meal to a clean 50-ml. flask; add 20 ml. of 30 per cent (by volume) alcohol. Shake for 10 minutes and filter or centrifuge. This extract should always be prepared on the day it is to be used, because on standing even in an icebox it will develop ammonia and will yield too high results. One should therefore not use more extract or a stronger extract than is really necessary. Koch (*J. Lab. Clin. Med.*, **11,** 776 (1926)) obtains a stable and active urease preparation by making a 75 per cent glycerol extract of jack bean meal.

Urease Paper. Transfer to a clean 200-ml. flask 30 g. of jack bean meal and 100 ml. of dilute alcohol (30 ml. of 95 per cent alcohol diluted to 100 ml.). Add 1 ml. of the buffer mixture described above. Stopper tightly and shake vigorously for at least five minutes and then shake less hard for about 10 minutes. Filter, or preferably centrifuge half an hour in 15-ml. tubes, the mouths of which have been covered with tinfoil. Transfer the extract to a porcelain dish and at once take it up on strips of rather heavy filter paper, Schleicher and Schüll, No. 597, and hang these up to dry over two threads about 15 cm. apart. While drying, the papers should not be exposed to air currents, for blasts of air seem to destroy the enzyme so long as water is present. As soon as the paper strips are thoroughly dry, cut them up into pieces about 1 cm. by 2.5 cm. and preserve in wide-mouth bottles. These urease papers will retain their activity for many months and even for years. The urease becomes fixed in the paper and it is only by shaking the solution several times during the digestion that one secures adequate contact and quantitative hydrolysis of the urea.

Antibumping Tube. As illustrated in Fig. 146. Made preferably from pyrex glass, 2 mm. in diameter at open end. May be obtained from Fisher Scientific Co., New York.

Antifoaming Oil Mixture. To one volume of crude fuel oil add about 10 volumes of toluene.

Saturated Borax Solution. Dissolve about 40 g. of reagent-grade sodium tetraborate (borax) in 1 liter of boiling water and allow to cool to room temperature. If unsaturated, add more borax and again heat.

0.1 N Acid. Either hydrochloric or sulfuric may be used, and it need not be standardized.

Standard Ammonium Sulfate Solution. Prepare a stock standard solution as follows: Dis-

Add 2 drops of acetate buffer solution and either 1 ml. of urease solution (prepared the same day) or a piece of urease paper. Insert a cork and then either let stand at room temperature for 25 minutes or immerse for 10 minutes in 700 ml. of water, having an initial temperature of about 45° C. Longer digestion does no harm. If urease paper is used the tube must be shaken occasionally during the digestion period. Cool the tube if warm and add an antibumping tube, 2 drops of antifoaming oil mixture, and 2 ml. of saturated borax solution. Connect at once with the delivery tube and a test tube receiver graduated at 25 ml. as shown in Fig. 146. The receiver contains 1 ml. of 0.1 N acid and 1 ml. of water. Fasten the boiling tube in a clamp and start the distillation by applying the flame of a microburner, preferably surrounded by a shield to prevent fluctuation of the flame due to air currents. As soon as the contents are nearly boiling, reduce the flame partially so that the first minute of boiling is very gentle. Then boil briskly for about three minutes and finally another minute with the delivery tube slightly raised from the surface of

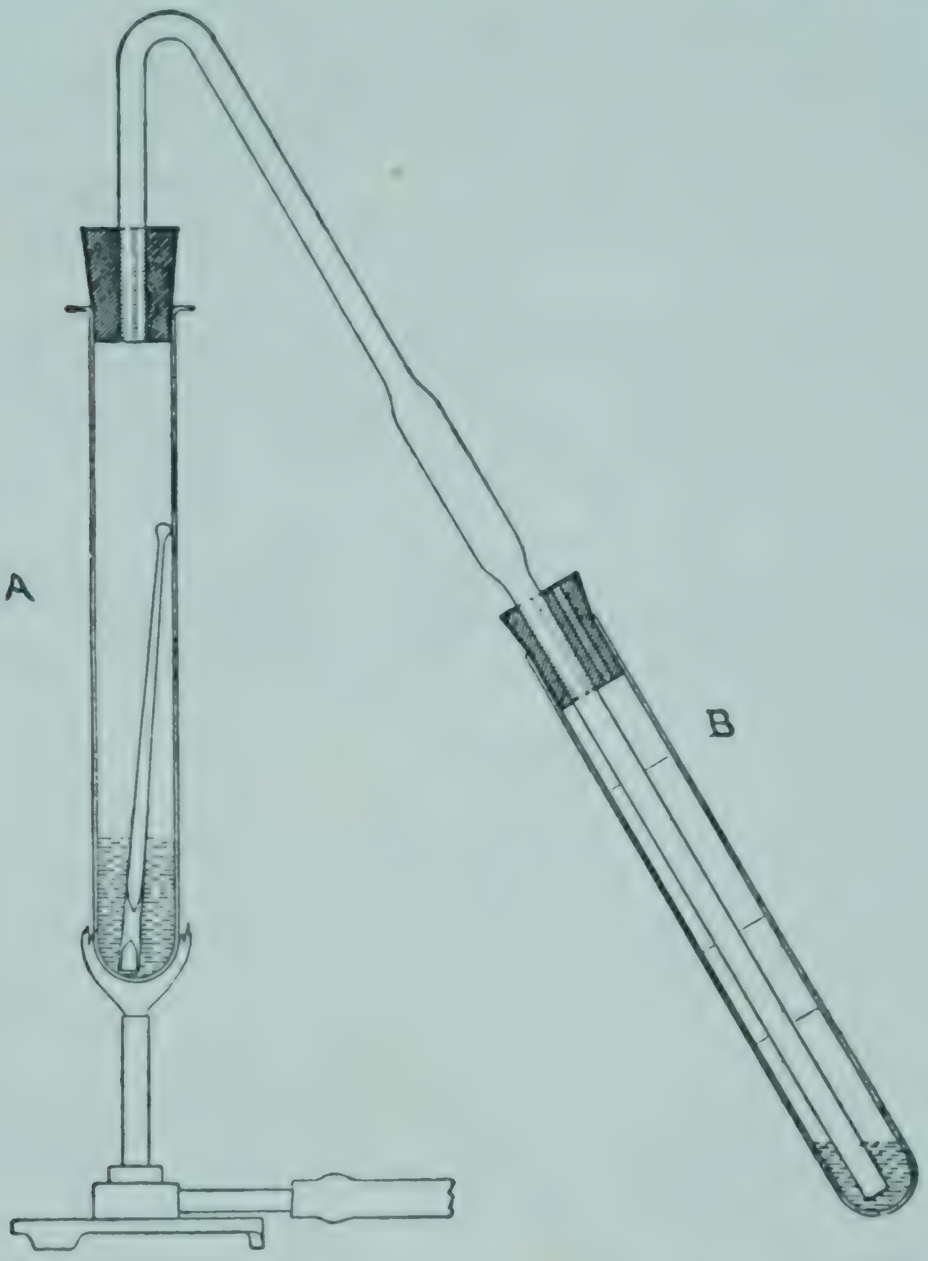

FIG. 146. MICRODISTILLATION APPARATUS FOR UREA DETERMINATION.

Note antibumping tube inside Tube *A* (Folin and Svedberg).

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solve 0.944 g. of dry reagent-grade ammonium sulfate in water, transfer with rinsings to a 1 liter volumetric flask, add a few drops of concentrated sulfuric acid, dilute to volume with water, and mix. This solution contains 1 mg. of nitrogen in 5 ml. and is stable indefinitely. To prepare the dilute standard used in the procedure, dilute 5 ml. of stock standard to 100 ml. with water. This solution contains 0.1 mg. of nitrogen in 10 ml., and is prepared fresh daily.

liquid in the receiver. To another test tube like the receiver, transfer 10 ml. of standard ammonium sulfate solution (containing 0.1 mg. of N), and 1 ml. of 0.1 N acid. Dilute both to a volume of about 20 ml.,[35] add 2.5 ml. of Nessler solution, dilute to the mark, mix and make the color comparison, using either a colorimeter or a photometer. For photometric measurement, prepare a blank tube containing 1 ml. of 0.1 N acid, water to about 20 ml., add 2.5 ml. of Nessler solution, dilute to 25 ml. and mix. Set the photometer to zero density with the blank, and determine the densities of standard and unknowns as described for the determination of nonprotein nitrogen on p. 547.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.1 \times \frac{100}{0.5} = \text{mg. urea N per 100 ml. blood}$$

The standard is satisfactory for blood values between 8 and 40 mg. per cent. For higher values, repeat the determination with less filtrate plus water.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.1 \times \frac{100}{0.5} = \text{mg. urea N per 100 ml. blood}$$

Under the photometric conditions specified on p. 547, the standard has a density of roughly 0.300. Up to 60 mg. per cent blood urea N may be measured satisfactorily. For higher values, or with cuvettes of greater depth than 1 cm., the analysis is carried out on less filtrate plus water, and the calculations corrected accordingly.

For separation of ammonia by aeration rather than distillation, see the next procedure.

Interpretation. Normally, the urea nitrogen of whole blood varies between 10 and 15 mg. per 100 ml. On a restricted hospital diet, however, values below 20 mg. should not be regarded as abnormal. In early nephritis the urea nitrogen may rise to 30 or 40 mg., but in the terminal stages of chronic nephritis and in some cases of acute nephritis marked urea retention may occur. High values may also be found in other conditions associated with damaged renal function, such as mercury bichloride poisoning, double polycystic kidney, intestinal obstruction, prostatic obstruction (in which case the urea N constitutes a valuable guide to surgical risk), lead poisoning, certain infections, cardiac failure, and so on. Relatively low figures for urea nitrogen are found in nephrosis (nonhemorrhagic nephritis with edema), which is probably of metabolic rather than renal origin.

The blood urea concentration has greater significance when interpreted with relation to urea excretion, as in the urea clearance test (Chapter 31).

2. *Method of Van Slyke and Cullen:*[36] *Principle.* Whole blood is treated with urease under optimal conditions for conversion of the urea to ammonium carbonate. The mixture is then made alkaline with potassium carbonate and aerated. In the original procedure, the aerated ammonia is absorbed in standard acid and the acid back-titrated with standard alkali. In the modification described here, the ammonia is absorbed in boric acid solution and titrated directly with standard acid.

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[35] In the original procedure of Folin and Svedberg, 1 ml. of gum ghatti solution (see Appendix) is added at this point. This is ordinarily not necessary and its use should be avoided if possible, since it decreases color intensity and alters the relationship between color intensity and concentration. If used, it must be added to both standard and unknown.

[36] Van Slyke and Cullen: *J. Biol. Chem.*, **11**, 211 (1914); **24**, 117 (1916).

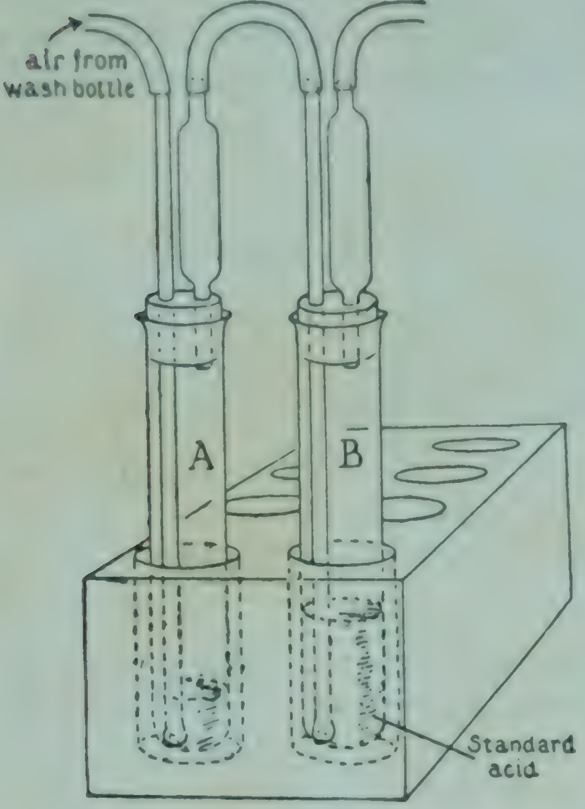

FIG. 147. VAN SLYKE AND CULLEN AERATION APPARATUS.

Procedure.[37] **Into a large test tube (200 × 28 mm., preferably thick-walled and lipless)[38] place 2 ml. of phosphate buffer, 5 drops of caprylic alcohol, and 0.5 ml. of 10 per cent urease solution. Add exactly 2 ml. of blood, mix gently, stopper loosely with a rubber stopper carrying the long inlet tube and the short outlet tube shown in Fig. 147, and allow to stand at room temperature for 10 minutes. This is Tube *A*. During the time of standing, prepare Tube *B* by placing 25 ml. of boric acid solution containing indicator in a second large test tube similar to the first, add a drop of caprylic alcohol, and stopper tightly with a rubber stopper likewise carrying inlet and outlet tubes.[39] Place in a test-tube rack or wooden block alongside Tube *A*, and connect the long inlet tube of *B* with the short outlet tube of *A* by means of a short length of rubber tubing,[40] as shown. When the 10-minute period is up, raise the stopper of *A* and add 5 ml. of a saturated solution of potassium carbonate down the side of the tube. Immediately replace the stopper and tighten it firmly in place. Attach the long inlet tube of *A* by rubber tubing to a large test tube or wash bottle half-full of N sulfuric acid, plus a few drops of caprylic alcohol,**

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[37] Reagents Required: *Phosphate Buffer.* Dissolve 6 g. of monopotassium phosphate (KH_2PO_4) and 2 g. of anhydrous disodium phosphate (Na_2HPO_4) in water, dilute to 1 liter, and mix. Add a few drops of chloroform as preservative.

Caprylic Alcohol. Dispense from a dropping bottle.

10 Per Cent Urease Solution. Stir up 1 g. of urease powder in a little water, sufficient to make a paste, and then add water, a little at a time, with stirring, to make the final volume 10 ml. Keep in the refrigerator when not in use, and make up only enough for each day's use, discarding the remainder, if any. "Arlco" urease (Arlington Chemical Co., Yonkers, N.Y.) may also be used. The activity of each bottle of urease should be tested by running through the standardization procedure on a urea solution as described below.

Boric Acid Solution, 2 Per Cent, with Indicator. Dissolve 20 g. of reagent-grade boric acid in about 500 ml. of hot water, cool, add 2 ml. of 0.1 per cent bromocresol green in alcohol (the aqueous solution of the sodium salt may also be used), dilute to 1 liter, and mix. This solution need not be prepared accurately, and may be prepared in large volume sufficient for several weeks' use. If the color fades on standing, more indicator may be added to the remaining portion. Only bromocresol green indicator may be used, since in the presence of caprylic alcohol other indicators (e.g., methyl red) are sufficiently altered in tint so that comparison of the end point with a control, as described in the text, becomes impossible. The 2 per cent strength of boric acid is recommended rather than 4 per cent because of the better end point and because it has been found capable of absorbing all the ammonia likely to be present, even in an analysis of urine (see Chapter 31).

Saturated Potassium Carbonate Solution. To 900 g. of pure dry potassium carbonate add 1 liter of water. Stir to dissolve as completely as possible.

Standard Sulfuric Acid Solution. This is to be of such strength that 1 ml. represents 0.2 mg. of nitrogen. Theoretically, a 0.0143 N solution is required. Practically, it is better to standardize the acid against pure urea as described here and adjust to the required strength. Measure exactly 15.0 ml. of N sulfuric acid from a buret into a 1-liter volumetric flask, dilute to the mark with water, and mix. Prepare a standard urea solution containing 1.07 g. of pure dry urea in 1 liter. Analyze a 2-ml. portion of the urea solution by the procedure described in the text, replacing the blood by the urea solution, and titrating with the acid. Multiply

and so arranged that when air is drawn through *A* it must first bubble through the wash bottle, thus freeing it of any ammonia. Attach the short outlet tube of *B* by rubber tubing to a suitable source of suction (water pump or vacuum line). When everything is ready, turn on the suction carefully and draw air slowly through *A* and *B* for about one minute, then increase the rate of air passage until the air is passing through as rapidly as possible. Continue the aeration for one hour. At the end of this time, slow down the rate of air passage (but do not stop it entirely) and disconnect the tubes, starting at the point farthest removed from the source of suction, to prevent any effects of back pressure. Remove the stopper and tubes from *B*, rinse down the long tube with a little water, and place the stopper aside. Titrate the contents of *B* with the standard sulfuric acid, preferably using a buret of 10 ml. capacity. The end point is when the more or less blue solution is exactly restored to its original yellow-green shade. This is best seen by having a control tube similar to *B* and containing 25 ml. of the boric acid–indicator solution. The contents of *B* are titrated until they exactly match the color of the control solution. The end point is usually sensitive to about 0.02 ml. of the standard acid.

CALCULATION. The strength of the acid is such that 1 ml. represents 0.2 mg. of nitrogen. Since 2 ml. of blood are used for analysis, the calculation is as follows:

$$\text{ml. standard acid required} \times 10 = \text{mg. urea N per 100 ml. blood}$$

As many pairs of tubes as desired may be connected in series as described, thus permitting the carrying out of a number of determinations at the same time. According to Van Slyke and Cullen, 75 liters of air will remove the ammonia satisfactorily from a solution half-saturated with potassium carbonate. The time required for complete aeration thus depends upon the rate of air passage, and may be shorter than that specified. It may be established by treating portions of standard ammonium sulfate solution with an equal volume of saturated potassium carbonate solution and aerating into boric acid for varying periods of time, testing for completeness of recovery of the ammonia by titration as described.

The aeration inlet tubes should have a perforated bulb at the tip, as shown, to break up the air, but this is not absolutely necessary. If the blood foams excessively and more caprylic alcohol is of no value, a few ml. of ethyl alcohol may be added to the mixture.

Interpretation. See previous method.

Variations of the Aeration Method. The aerated ammonia may be absorbed in 25 ml. of 0.01 N sulfuric acid containing alizarin or methyl red indicator, and the excess acid titrated with 0.01 N sodium hydroxide,

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15.0 by the number of ml. of acid used in the titration and divide the result by 5.00 to find the exact volume of N acid which must be diluted to 1 liter for the correct strength. Thus if 4.77 ml. of acid were used in the titration, $\frac{15.0}{5.00} \times 4.77 = 14.3$ ml. Discard the first lot of acid and prepare a second using the correct volume of N sulfuric acid. Check by repeating the standardization; exactly 5.00 ml. of acid should be required in the titration. The acid solution is stable indefinitely and may be made up in large volume at one time.

[38] Both Kimble No. 49050 and Corning No. 7980 are satisfactory.

[39] It is good practice to have marked fitted stoppers for the *A* and *B* tubes separately and not to interchange them, thus avoiding the possibility of error due to traces of carbonate on an *A* inlet tube entering the boric acid of a *B* tube.

[40] Thick-walled stethoscope tubing is satisfactory. Cut in 6-inch lengths, scrub with a buret brush, then boil for 30 minutes in 10 per cent sodium hydroxide solution, making sure that the tubes are filled with the solution. Rinse thoroughly in tap water and distilled water. Do not use acid for washing the tubes.

as described in the original procedure of Van Slyke and Cullen. The aeration procedure may also be applied to the Folin-Wu filtrate after urease treatment, the liberated ammonia being absorbed in dilute acid and determined colorimetrically. The ammonia aerated from urease-treated whole blood may also be determined colorimetrically instead of by titration.

3. ***Method of Karr:***[41] ***Principle.*** By the action of urease, the urea in a protein-free filtrate is converted to ammonium carbonate which is nesslerized in the presence of a protective colloid. The interference of peptones and amino acids (Folin and Wu) is regarded as so slight and uniform as not to influence the clinical value of the results.

Procedure.[42] **Transfer 5 ml. of Folin-Wu blood filtrate to a test tube, and in a similar tube place 5 ml. of standard urea solution, containing 0.075 mg. of nitrogen. To each tube add 0.5 ml. of buffer solution and 5 drops of urease solution. Place in a bath at 50° C. for 15 minutes. Transfer the contents of each tube, with rinsings, to separate test tubes[43] graduated at 22.5 and 25 ml., and dilute with water to the 22.5-ml. mark. Add 3 drops of gum ghatti solution, followed by Nessler solution to the 25-ml. mark. Mix, allow to stand for 10 minutes, and read within the next 20 minutes, using either a colorimeter or photometer. For photometric measurement, follow the conditions specified on p. 547 for the determination of nonprotein nitrogen, setting the photometer to zero density with a blank prepared by treating 5 ml. of water with buffer, urease, etc., exactly as described above for a blood filtrate.**

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.075 \times \frac{100}{0.5} = \text{mg. urea N per 100 ml. blood}$$

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.075 \times \frac{100}{0.5} = \text{mg. urea N per 100 ml. blood}$$

Interpretation. See under method of Folin and Svedberg above.

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[41] Karr: *J. Lab. Clin. Med.*, **9**, 3 (1924). The use of gum ghatti as a stabilizing colloid by Folin (*J. Biol. Chem.*, **81**, 231 (1929) in his sugar method (p. 575) suggested its application in the urea method to Looney (*J. Biol. Chem.*, **88**, 189 (1930).

[42] Reagents Required: *Standard Urea Solution.* Dissolve 0.3215 g. of pure dry urea in water and dilute to 500 ml. in a volumetric flask. Add a little chloroform or toluene as a preservative. This solution contains 0.3 mg. of urea nitrogen per ml. To prepare the working standard, dilute 5 ml. of stock standard to 100 ml. with water in a volumetric flask, and mix. Prepare fresh daily. This solution contains 0.075 mg. of urea nitrogen in 5 ml.

Buffer Solution. Dissolve 20 g. of crystalline sodium acetate in water, add 2.2 ml. of 10 per cent acetic acid, dilute to 100 ml. with water, and mix. Add a little toluene or chloroform as preservative.

Urease Solution. Place 15 g. of jack bean meal (obtainable from the Arlington Chemical Co., Yonkers, N.Y.), about 2 g. of "Permutit" (see Appendix), 16 ml. of 95 per cent alcohol, and 84 ml. of water in a 200-ml. flask. Shake more or less continuously for about 15 minutes. Pour onto a filter and allow to filter overnight in the refrigerator. Keep the filtrate in the cold, transferring a portion to a dropping bottle for daily use. Prepare fresh every three to four weeks, or when a blank analysis shows the presence of significant amounts of ammonia.

Gum Ghatti Solution. See Appendix.

Nessler Solution. The Koch-McMeekin preparation is recommended (see Appendix).

[43] Separate tubes for nesslerization are necessary because of the "poisoning" action of mercury on urease. When this is suspected in the conversion tubes they should be cleaned with strong nitric acid.

4. Method of Leiboff and Kahn:[44] ***Principle.*** The urea in the Folin-Wu blood filtrate is converted to ammonia by acid hydrolysis under pressure and directly nesslerized. The slight conversion of nonurea substances to ammonia is unimportant clinically. By the elimination of urease, turbidity after nesslerization is claimed to be avoided even with filtrates high in urea.

DETERMINATION OF CREATININE

Introduction. Blood creatinine is ordinarily determined by reaction in a protein-free filtrate with alkaline picrate to form a red color (the Jaffé reaction), which is then compared with a standard. A color reaction of creatinine with dinitrobenzoate has also been described.[45] It is well recognized that the Jaffé reaction is by no means specific for creatinine, and that other substances are present in blood (chiefly in the red cells) which contribute to the color, so that results on whole blood filtrates are undoubtedly too high.[46] Practically all of the chromogenic material in plasma, however, appears to be creatinine, and therefore plasma is preferred to whole blood for analysis. Most of the results in the literature have been obtained on whole blood. A specific method for creatinine determination appears to be the measurement by the Jaffé reaction before and after treatment with a bacterial preparation which destroys creatinine.[47]

Method of Folin and Wu:[48] ***Principle.*** A portion of the blood filtrate is treated with alkaline picrate solution and the color developed is compared in a colorimeter or photometer with that produced by a known amount of creatinine under the same conditions.

Procedure.[49] **Transfer 10 ml. of 1:10 tungstic acid filtrate of whole blood or plasma (preferably the latter) to a small flask or test tube. In a second container, place 5 ml. of standard creatinine solution, containing 0.03 mg. of creatinine, and add 15 ml. of water. Add 5 ml. of freshly prepared alkaline picrate reagent to the blood filtrate, and 10 ml. to the diluted creatinine**

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[44] Leiboff and Kahn: *J. Biol. Chem.*, **83**, 347 (1929).

[45] Benedict and Behre: *J. Biol. Chem.*, **114**, 515 (1936); Langley and Evans: *ibid.*, **115**, 333 (1936).

[46] Hunter and Campbell: *J. Biol. Chem.*, **32**, 195 (1917); Behre and Benedict: *ibid.*, **52**, 11 (1922); **117**, 415 (1937); Miller and Dubos: *ibid.*, **121**, 447, 457 (1937); Gaebler and Abbott: *ibid.*, **123**, 119 (1938).

[47] Miller and Dubos: *loc. cit.;* Allinson: *J. Biol. Chem.*, **157**, 169 (1945).

[48] Folin and Wu: *J. Biol. Chem.*, **38**, 81 (1919). See also Peters: *J. Biol. Chem.*, **146**, 179 (1942); Bonsnes and Taussky: *ibid.*, **158**, 581 (1945).

[49] Reagents Required: *Standard Creatinine Solution.* Prepare a *stock standard* by dissolving 1 g. of pure dry creatinine in 0.1 N hydrochloric acid and diluting to 1 liter with the acid. This solution is stable indefinitely, and contains 1 mg. of creatinine per ml. To prepare the *working standard,* transfer 3 ml. of stock standard, containing 3 mg. of creatinine, to a 500-ml. volumetric flask, add 50 ml. of 0.1 N hydrochloric acid, dilute with water to 500 ml., and mix. This standard contains 0.03 mg. of creatinine in 5 ml., and is stable for a week or more if preserved by the addition of a few drops of toluene.

Alkaline Picrate Reagent. (*a*) Prepare a saturated solution of purified picric acid (see Appendix). It is essential that this solution be *saturated,* otherwise serious error may result. A 10-ml. portion, titrated with 0.1 N alkali in the presence of phenolphthalein as indicator, should require 5.2 to 5.4 ml. of alkali for neutralization. (*b*) To prepare the fresh alkaline picrate reagent, transfer 25 (or 50) ml. of the saturated picric acid solution to a flask, add 5 (or 10) ml. of 10 per cent sodium hydroxide solution, and mix. Use within a short time after preparing. On standing, crystals may form in the solution which do not impair its effectiveness but render measurement difficult.

standard. Mix, and allow to stand for 15 minutes for complete color development. Read in the colorimeter or photometer within the next 15 minutes.

For colorimetric measurement, first match the standard against itself carefully, and then compare with the unknowns in the usual way. For photometric measurement, transfer the solutions to suitable containers and determine the densities in the photometer at 520 mμ (see Fig. 148). Set the photometer to zero density with water alone. Determine the density of a blank prepared by treating 10 ml. of water with 5 ml. of the alkaline picrate reagent, and subtract this value from the observed densities of standard and unknown to obtain their true values.

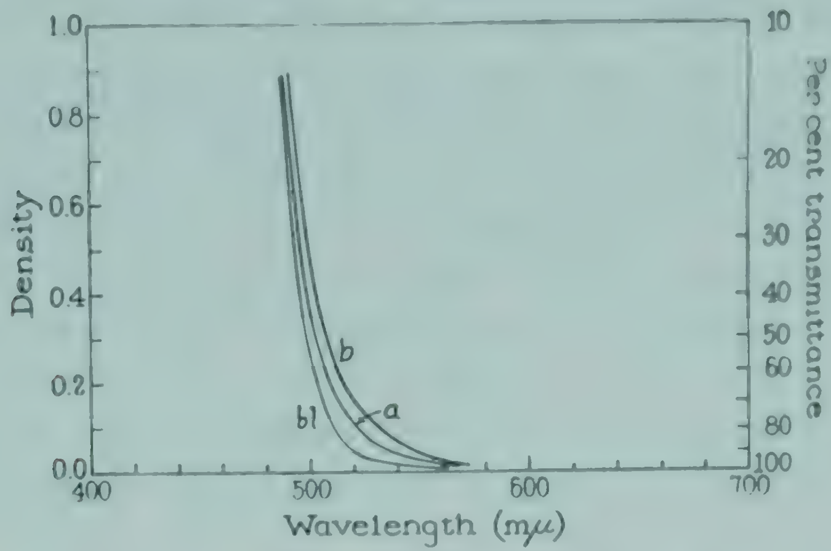

FIG. 148. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN FOLIN-WU BLOOD CREATININE METHOD.

Alkaline picrate alone (*bl*); alkaline picrate plus 0.03 mg. creatinine (*a*); plus 0.06 mg. creatinine (*b*). Solution depth, 1 cm.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. creatinine in standard} \times \frac{100}{1} \times \frac{15}{30}$$
$$= \text{mg. creatinine per 100 ml. blood or plasma}$$

In connection with this calculation, it is to be noted that the standard is made up to twice the volume of the unknown, so that a volume correction factor ($^{15}/_{30}$) is introduced. The standard specified corresponds to a blood creatinine of 1.5 mg. per cent, and is satisfactory for the range of 1 to 2 mg. per cent *only*, because of the presence of the high blank color of the alkaline picrate and the significant deviation from Beer's law (see p. 557) shown by this color reaction. It is good practice, therefore, to prepare several standards at different concentration levels, to provide for values outside the indicated range. If a high creatinine should be encountered without several standards ready, the determination may be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution which has first been diluted with two volumes of water, to preserve equality of picric acid and alkali concentration. It is better, however, to repeat the analysis if possible, using less filtrate plus water to 10 ml., correcting the calculations accordingly.

For photometric measurement, values up to about 5 mg. per cent blood creatinine may be calculated as follows:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. creatinine in standard} \times \frac{100}{1} \times \frac{15}{30}$$
$$= \text{mg. creatinine per 100 ml. blood or plasma}$$

For higher values, repeat the analysis with 5 ml. of filtrate plus 5 ml. of water, and multiply the results by 2. At 520 mμ and in a 1-cm. cuvette, the density of the standard described, corrected for the blank, is approximately 0.050.

This calculation is an *approximation* only, suitable for routine clinical purposes but not for precise work, since it is based on Beer's law, and the creatinine color does not follow Beer's law exactly at any concentration ordinarily encountered. For more exact work, a calibration curve relating observed densities to concentration must be prepared and results read from this curve. To obtain such a curve, prepare a standard creatinine solution which is twice as strong as that used in the procedure, by transferring 6 ml. of the stock 0.1 per cent creatinine solution to a 500-ml. volumetric flask, adding 50 ml. of 0.1 N acid, and diluting to the mark with water. This solution contains 0.06 mg. of creatinine in 5 ml. Transfer the following amounts of this solution (preferably in duplicate) to small flasks: 0.0, 5.0, 10.0, 15.0, and 20.0 ml. Add sufficient water to each flask to make the final volume 20 ml., and then add 10 ml. of alkaline picrate reagent to each. Mix, allow to stand 15 minutes, and determine the densities in the photometer under the conditions described in the text. The first flask is a blank; subtract its average value from the other average values to obtain their true densities. The amounts of creatinine in the various flasks are 0.06, 0.12, 0.18, and 0.24 mg., respectively, corresponding under the conditions of analysis to blood creatinine contents of 3, 6, 9, and 12 mg. per cent. Plot the true densities against the equivalent mg. per cent concentrations on cross-section paper and draw a smooth curve to include all the points. A curve similar to that shown in Fig. 149 should be obtained. From such a curve, the concentration of an unknown may be read off if its density has been established.

In using such a curve, it is important to remember that it is valid only if the analysis of an unknown is carried out under conditions similar to those prevailing at the time the curve was established. In general, the calibration for one photometer is not applicable to another instrument, even of the same make, and changing the wavelength setting or filter will influence the curve. The curve should be checked when a new lot of saturated picric acid[50] is prepared, although correcting for the blank as indicated will ordinarily take care of slight variations in the color of the alkaline picrate reagent. Time control and temperature control are important; the curve at 30° C. is not the same as at 20°. All of these precautions are necessary for obtaining accurate results.

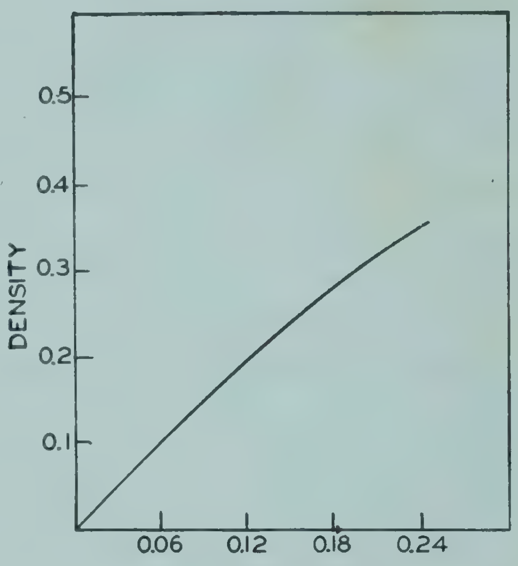

FIG. 149. TYPICAL CALIBRATION CURVE FOR BLOOD CREATININE DETERMINATION, AT 520 Mμ, 1 CM. SOLUTION DEPTH.

Interpretation. Creatinine is the least variable nitrogenous constituent of the blood, in which it exists to the extent of 1 to 2 mg. per 100 ml. of whole blood; the average value for plasma is nearer 1 mg. per cent. In early nephritis, values of from 2 to 4 mg. are noted, and in chronic hemorrhagic nephritis with uremia, 4 to 35 mg. Creatinine is more readily excreted by the kidneys than urea or uric acid, and an increase of creatinine to 4 or 5 mg. or over per 100 ml. is evidence of marked impairment of kidney function. Such high creatinine values in chronic hemorrhagic nephritis indicate an unfavorable prognosis, although high values may obtain in acute cases over long periods.

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[50] Peters (*loc. cit.*) recommends a picric acid solution containing 11.75 g. per liter, rather than a saturated solution, to minimize differences in solubility of picric acid at summer and winter temperatures.

DETERMINATION OF CREATINE

Introduction. The creatine of blood is ordinarily determined by the Folin-Wu procedure of heating the protein-free filtrate with acid, which converts creatine to creatinine. The total creatinine present is then determined by the procedure used for blood creatinine. The value for preformed creatinine, as established by separate analysis, is subtracted from the total creatinine value, to give the amount of creatine, expressed as creatinine. There is no satisfactory method for the direct determination of blood creatine. The specificity of the method based on acid treatment and the Jaffé reaction for creatinine is open to question. Increased specificity is undoubtedly obtained by the use of the creatinine-destroying bacteria of Miller and Dubos;[51] few applications of this principle to knowledge of blood creatine have as yet been made.

***Procedure.* Transfer 5 ml. of a 1:10 tungstic acid filtrate of whole blood to a test tube graduated at 25 ml. Add 1 ml. of normal hydrochloric acid. Cover the mouth of the test tube with tin foil and heat in the autoclave to 130° C. for 20 minutes or to 155° C. for 10 minutes. Cool. Add 5 ml. of freshly prepared alkaline picrate solution (as used for blood creatinine determination) and let stand for 8 to 10 minutes, then dilute to 25 ml. At the same time, prepare a standard creatinine solution by adding to 10 ml. of creatinine solution, containing 0.06 mg. of creatinine, in a 50-ml. volumetric flask, 2 ml. of normal acid, and 10 ml. of the alkaline picrate reagent, and after 10 minutes, diluting to 50 ml. Read in the colorimeter or photometer as described for the determination of blood creatinine (p. 556). The blank for photometric measurement is prepared by treating 10 ml. of water in a 50-ml. volumetric flask with 2 ml. of acid and 10 ml. of alkaline picrate reagent and diluting to 50 ml. with water.**

In the case of uremic bloods containing large amounts of creatinine, 1, 2, or 3 ml. of blood filtrate, plus water enough to make approximately 5 ml., are substituted for the 5 ml. of filtrate, and calculations corrected accordingly.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.06 \times \frac{100}{0.5} \times \frac{25}{50} = \text{mg. ``total creatinine'' per 100 ml. blood}$$

Subtract the preformed creatinine content, as determined by separate analysis (p. 555), from the "total creatinine," to obtain the creatine content, expressed as creatinine. The same precautions as were described for the determination of blood creatinine, concerning deviation from Beer's law and the use of several standards if necessary, must be observed.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.06 \times \frac{100}{0.5} \times \frac{25}{50} = \text{mg. ``total creatinine'' per 100 ml. blood}$$

Determine creatine by subtracting preformed creatinine value as described above.

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[51] Allinson: *J. Biol. Chem.*, **157**, 169 (1945). For application of the bacterial method to the determination of creatine in tissues, see Miller, Allinson, and Baker: *J. Biol. Chem.*, **130**, 383 (1939); Borsook and Dubnoff: *ibid.*, **132**, 559 (1940).

As with the determination of creatinine, the calculation given is only an approximation because of deviation from Beer's law, and for precise results a calibration curve similar to that described on p. 557 should be constructed and used. The calibration curve prepared for the preformed blood creatinine determination cannot be used here because of the presence of acid and the different proportion of alkaline picrate used. In preparing this curve, remember that only 5 ml. of filtrate are used for analysis instead of 10 ml. as in the creatinine determination, so that a given amount of creatinine in the standard corresponds to twice as high a total creatinine content per 100 ml. of blood as in the calibration for preformed creatinine. Thus the standard described, containing 0.06 mg. of creatinine in a final volume of 50 ml., is equivalent in an analysis to a total blood creatinine of 6 mg. per cent, with other amounts in proportion.

Interpretation. Blood creatine content as determined by this and other methods ranges between 3 to 7 mg. per 100 ml. of whole blood. The creatine of blood is found almost entirely in the red cells, variations in cell count therefore presumably influencing results. Except in the case of infants and pregnant women, the amount of creatine in plasma is quite small; Allinson (*loc. cit.*) reports values ranging from 0.4 to 0.8 mg. per cent.[52] No significant variations have as yet been associated with pathological conditions, so the determination has little clinical value at the present time. Increases have been noted after severe muscular injury and following experimental removal of the kidneys.

DETERMINATION OF URIC ACID

Introduction. Blood uric acid is ordinarily determined by colorimetric procedures based upon the reaction between uric acid and certain complex phosphotungstic acid reagents (or their equivalent), usually in the presence of cyanide,[53] to form a blue color. Procedures utilizing the reducing action of uric acid on ferricyanide have also been described.[54] The determination of blood uric acid has been the subject of much criticism and study. It appears probable that a portion of the blood uric acid is lost during precipitation of the proteins; up to now, there appears to be no simple way to avoid this.[55] The use of the various color reagents on a blood filtrate directly, without preliminary separation of interfering material, is felt by many investigators to be unsound, because of both the nonspecificity of the color reaction and the possible presence of substances which inhibit color development with uric acid itself. A combination of these effects (one tending to increase color, the other decreasing it) is felt by some to be responsible for the apparently satisfactory results obtained in some procedures. Interfering material appears to be present largely in the red cells of blood; the use of plasma or serum for analysis is therefore gaining ground and is to be preferred. Increased specificity is also obtained by separating the uric acid from interfering material prior to analysis. Color development before and after treatment with preparations of the enzyme *uricase*, which oxidizes uric acid, has also been proposed as a basis of specificity.[56] Of the three procedures described below, two are based on separation of interfering material before color development. The third, the method of Brown, is a direct method which appears to be as satisfactory as any that have been described, and simpler than most.

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[52] See also Tierney and Peters: *J. Clin. Invest.*, **22**, 595 (1943).

[53] For a procedure not involving the use of cyanide, see Kern and Stransky: *Biochem. Z.*, **290**, 419 (1937).

[54] See Bulger and Johns: *J. Biol. Chem.*, **140**, 427 (1941).

[55] See, however, Kern and Stransky (*loc. cit.*).

Method of Folin (Isolation Procedure):[57] ***Principle.*** Uric acid is precipitated as silver urate, directly from the blood filtrate. The uric acid is set free by means of acid chloride solution and determined colorimetrically or photometrically after the addition of phosphotungstic acid, which gives a blue solution.

Procedure.[58] **Transfer 5 ml. of the blood filtrate[59] to a centrifuge tube. Add 2 ml. of the acid silver solution. Centrifuge at once. All the uric acid, down**

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[56] Blauch and Koch: *J. Biol. Chem.*, **130**, 443 (1939). For a description of a uricase procedure applied to the determination of uric acid in urine, see Chapter 31, "Urine: Quantitative Analysis."

[57] Folin: *J. Biol. Chem.*, **101**, 111 (1933); **106**, 311 (1934). The Folin "direct method" is similar to the procedure described here and requires the same reagents, except that the precipitation with acid silver solution is omitted. A 5-ml. portion of the blood filtrate is treated directly with the cyanide-urea solution, uric acid reagent, etc., exactly as described in the text. Calculations and directions for photometric and colorimetric measurement are the same.

[58] Reagents Required: *Acid Silver Solution.* To 5 ml. of 85 per cent lactic acid add 100 ml. of water and 5 g. of Na_2CO_3 and boil. Dissolve 25 g. of silver nitrate in about 700 ml. of water, add the partly neutralized lactic acid solution, and dilute to 1 liter. After a few days' exposure to sunlight and filtering, this reagent keeps fairly well. When used only occasionally, however, it should be filtered before using.

Standard Uric Acid Solution. The solution made as follows will keep for at least five years. Weigh out on a watch glass exactly 1 g. of uric acid and transfer it to a liter volumetric flask by means of a not too small, dry funnel. Tap the funnel, so as to transfer nearly the whole of the uric acid to the flask. Transfer 0.6 g. of lithium carbonate to a 250-ml. Florence flask, add 150 ml. of water; shake about five minutes until dissolved. Some insoluble material remains, and it is usually best to filter. Heat the solution or filtrate to 60° C. Also, warm the liter flask under running warm water. Pour the warm lithium carbonate solution into the liter flask, incidentally washing into it the traces of uric acid which adhered to the watch glass and funnel. Shake so as to dissolve the uric acid promptly. A little additional warming under hot tap water is permissible. The lithium carbonate solution is not always perfectly clear even when filtered, and one should not mistake this little turbidity for undissolved uric acid and keep warming and shaking too long. In five minutes all of the uric acid should be dissolved. Shake the flask under cold running water without undue delay. Add 20 ml. of 40 per cent formalin, and half fill the flask with distilled water. Finally add, from a pipet, rather slowly and with shaking, 25 ml. of normal sulfuric acid. Dilute to volume, mix thoroughly, and transfer to a clean, tightly stoppered bottle. This stock solution, containing 1 mg. of uric acid per ml., should be kept away from light.

To prepare the working standard, dilute 1 ml. of the stock solution, with water only, to 250 ml. It behaves exactly like a lithium carbonate solution of uric acid and keeps perfectly for many days. (5 ml. = 0.02 mg. uric acid.)

Urea-Cyanide Solution (Poisonous!). Transfer 75 g. of Merck's Blue Label sodium cyanide to a 2-liter beaker, add 700 ml. of water, and stir until the cyanide is completely dissolved. Add 300 g. of urea and stir. Then add 4 to 5 g. of calcium oxide and stir for about 10 minutes. Filter, at once if necessary for immediate use, but preferably not until the next day. To the filtrate add about 2 g. of powdered lithium oxalate, shake occasionally for 10 to 15 minutes, and filter.

Uric Acid Reagent. Transfer 100 g. of sodium tungstate (free from molybdate, cf. footnote 59 below) to a 500 ml. Florence flask. Mix 32 to 33 ml. of 85 per cent phosphoric acid with 150 ml. of water. Pour the resulting solution on to the tungstate and mix. Add a few pebbles and boil very gently over a microburner for 1 hour. Loss of liquid during the boiling is prevented by using, as a condenser, a funnel holding a 200-ml. flask filled with cold water. At the end of the boiling period decolorize with a little bromine water, boil off the excess bromine, cool, and dilute to 500 ml.

If the reagent so obtained is not perfect (in other words, if it gives a blank with Merck's urea-cyanide or with urea-cyanide plus tyrosine), add 3 to 5 g. of sodium tungstate (but

to the last trace, will now be in the precipitate. Decant the supernatant solution as completely as possible, and add 1 ml. of a 10 per cent solution of sodium chloride in 0.1 N hydrochloric acid. Stir *thoroughly* with a fine glass rod; add 4 ml. of water, and stir again. Centrifuge. Pour the supernatant solution as completely as possible into a test tube graduated at the 25-ml. mark. Remove the last drop by touching the lip of the centrifuge tube to the side of the graduated test tube. Prepare two standards by placing 3 ml. (plus 2 ml. of water) and 5 ml. respectively of standard uric acid solution, containing 0.02 mg. of uric acid in 5 ml., in separate test tubes, and for photometric measurement prepare a blank tube containing 5 ml. of water only. To each tube add 10 ml. of urea-cyanide solution (*Poisonous! from a buret*) and mix well by lateral shaking. Add 4 ml. of the uric acid reagent to each tube, mix well by lateral shaking, and note the time. Let stand for 20 minutes, dilute to the 25-ml. mark with water, stopper, and mix by inversion. Read in the usual way within the next half-hour in the colorimeter or photometer. For photometric measurement, determine the densities at 420 mμ, setting the photometer to zero density with the blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. uric acid in standard} \times \frac{100}{0.5}$$
$$= \text{mg. uric acid per 100 ml. blood or plasma}$$

Read the unknown against the standard which most closely matches it on inspection, and substitute the proper value for the uric acid content of the standard (0.012 or 0.020, as the case may be) in the calculation formula above. The stronger standard corresponds to a blood with 4 mg. per cent uric acid; up to 8 mg. per cent may be read satisfactorily. For higher values, instead of using the entire supernatant from the acid extraction of the precipitated silver urate as described, pipet out 2.5 ml. at this point, add 2.5 ml. of water, and continue with the addition of urea-cyanide, color development, etc., as described, multiplying the final results by 2.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. uric acid in standard} \times \frac{100}{0.5}$$
$$= \text{mg. uric acid per 100 ml. blood or plasma}$$

Either standard may be used, therefore only one is necessary. At 420 mμ, and in a 1-cm. cuvette, the density of the stronger standard, corresponding to 4 mg. per cent blood uric acid, is approximately 0.500. Up to 8 mg. per cent may be accurately read under these conditions. For higher values, or with deeper cuvettes, use half of the supernatant fluid from the acid extraction as described above under colorimetric measurement, and multiply the results by 2.

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no more) and boil for another 10 to 15 minutes, then cool, and decolorize as before. The addition of a little extra tungstate and the short second boiling can also be made without first testing the reagent for a blank.

[59] The tungstic acid deproteinization preceding estimation of uric acid by this method must be made with sodium tungstate entirely free from molybdate. A product meeting this specification is made by the Mallinckrodt Chemical Co. The absence of molybdate should be confirmed on each new batch of sodium tungstate by applying the following xanthate test (Folin and Trimble: *J. Biol. Chem.*, **60**, 473 (1924)): Dissolve 1 g. of sodium tungstate in 5 to 10 ml. of water. The solution should be alkaline. Add about 0.2 g. of solid potassium ethyl xanthate (Eastman, or prepare as directed in original paper). Shake until dissolved. Add dropwise with shaking, 20 per cent H_2SO_4, until the curdy tungstic acid precipitate dissolves. In the presence of molybdenum, as the solution becomes definitely acid, a pink to deep plum color forms. The colored compound, molybdenum xanthate, is soluble in chloroform.

Interpretation.[60] Normal human whole blood usually contains from 2 to 3.5 mg. of uric acid per 100 ml. by this method. The normal range for plasma is 3 to 5 mg. per cent. In early nephritis values of from 3 to 10 mg. may be noted, and in advanced cases values as high as 25 mg. are observed. The determination of blood uric acid is generally regarded as an unsatisfactory index of renal impairment since its level may be affected by a chemical destructive process as well as by excretion, which might account for the observed inconsistencies between hyperuricemia and other nonprotein nitrogen retention.

In gout, high uric acid values (4 to 10 mg.) are usually found. Determination of uric acid is of *diagnostic value* in gout prior to the stage of tophi formation, particularly since normal values, in the absence of salicylate or dietary therapy, would ordinarily exclude a positive diagnosis. However, uric acid is similarly increased in early nephritis and many cases of gout with high uric acid values also show defective kidney function by other tests. The same difficulty is met with in considering the high values (2 to 8 mg.) obtained in other arthritic conditions, usually associated with increases in urea also. The existence of nephritis in such cases has not been entirely excluded and many typical cases of arthritis show values below 3 mg. Salicylates and atophan (cincophen) tend to reduce the uric acid content of the blood.

The excessive breakdown of nuclear material in leukemia is accompanied by elevated uric acid values. In addition to the numerous conditions resulting in renal damage, such as mercury or lead poisoning, eclampsia, acute infections, malignancy, etc., the blood uric acid is also increased in certain cardiac conditions which ultimately involve the kidneys.

2. Method of Newton:[61] ***Principle.*** Interfering material in the blood filtrate is removed by the acid silver chloride precipitation method of Benedict and Behre.[62] The uric acid remaining is determined colorimetrically by reaction at room temperature in the presence of cyanide with a special arsenotungstate reagent.

Procedure.[63] **The blood proteins are precipitated with the molybdotungstic acid reagent of Benedict and Newton rather than by the Folin-Wu tungstic acid method, although this latter filtrate may also be used. Transfer 1 volume of blood to a small flask and add 7 volumes of water to lake. Add 1 vol-**

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[60] See reviews by Folin, Berglund, and Derick: *J. Biol. Chem.*, **60**, 361 (1924), and Rose: *Physiol. Revs.*, **3**, 544 (1923). See also Myers and Muntwyler: *Ann. Rev. Biochem.*, **9**, 303 (1940).

[61] Newton: *J. Biol. Chem.*, **120**, 315 (1937).

[62] Benedict and Behre: *J. Biol. Chem.*, **92**, 161 (1931). For the complete uric acid procedure of these authors, see the eleventh edition of this book.

[63] Reagents Required: *For Protein Precipitation: Tungstomolybdate Solution.* Boil 10 g. of reagent-grade ammonia-free molybdic acid with 50 ml. of normal sodium hydroxide for 4 to 5 minutes. Filter, and wash the residue on the filter with 150 ml. of hot water. Cool the filtrate and washings and add to a solution of 80 g. of sodium tungstate dissolved in 600 ml. of water. Dilute to 1 liter and mix. *0.62 N Sulfuric Acid.* Dilute 620 ml. of N sulfuric acid to 1 liter with water, and mix. Both these solutions are stable indefinitely.

Acid Lithium Chloride. Dissolve 7.5 g. of lithium chloride in water, add 35 ml. of concentrated hydrochloric acid, dilute to 1 liter with water, and mix. Stable indefinitely.

Silver Nitrate Solution. Dissolve 29 g. of reagent-grade silver nitrate in water, dilute to 1 liter, and mix. Keep in a brown bottle.

"Acid Blank" Solution (for photometric measurement only). Dilute 7 ml. of concen-

ume of tungstomolybdate solution followed by 1 volume of 0.62 N sulfuric acid, with shaking. Allow to stand for a few minutes, then pour onto a dry filter and collect the filtrate in a small dry flask. For plasma or serum, use 8 volumes of water and 0.5 volume of the tungstomolybdate and acid.

Transfer 5 ml. of the 1:10 filtrate prepared as described to a 15-ml. centrifuge tube, add 1 ml. of acid lithium chloride, and mix. Add 1 ml. of silver nitrate solution and shake well. Centrifuge at once and pour the supernatant fluid[64] into a test tube, allowing time for complete drainage. Touch the lip of the centrifuge tube to the test tube to obtain the last drop. In a second test tube place 5 ml. of the standard uric acid solution, containing 0.02 mg. of uric acid, and add 2 ml. of water. For photometric measurement prepare a third tube containing 5 ml. of "acid blank" solution plus 2 ml. of water. To each tube add *from a buret* 3 ml. of the cyanide-urea reagent (*poisonous*), mix by lateral shaking, and follow with 1 ml. of the diluted lithium arsenotungstate reagent *from a buret* (*poisonous*). Stopper and mix by inversion. Allow to stand 10 minutes, and then read in a colorimeter or photometer. For photometric measurement, determine the densities at 520 mμ, setting the photometer to zero density with the blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.02 \times \frac{100}{0.5} = \text{mg. uric acid per 100 ml. blood or plasma}$$

With the standard at 15 mm., readings of the unknown between 10 mm. and 30 mm. are reliable. For higher values, use less filtrate plus water to 5 ml. in the acid silver chloride precipitation, and correct the calculations accordingly.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.02 \times \frac{100}{0.5} = \text{mg. uric acid per 100 ml. blood or plasma}$$

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trated hydrochloric acid to 1 liter with water and mix.

Cyanide-Urea Reagent (*Poisonous!*). Dissolve 100 g. of urea in 500 ml. of water. Add 25 g. of reagent-grade sodium cyanide and stir to dissolve. This solution is usable for 1 month or so. Fresh solution must be prepared if color develops in a blank test run as described for photometric measurement in the text.

Lithium Arsenotungstate Reagent (*Poisonous!*). Dissolve 100 g. of reagent-grade sodium tungstate in 500 ml. of water, and add 140 g. of reagent-grade arsenic pentoxide. Boil under a reflux condenser for 1 hour. Remove the condenser and continue boiling until the volume is reduced to about 200 ml. Pour this solution onto 100 g. of solid lithium chloride in a beaker. Stir until all of the lithium chloride has gone into solution. Chill the contents of the beaker to at least 10° C. for 2 hours but no longer. Filter off the precipitated lithium arsenotungstate by suction and suck as dry as possible. Excess water may also be removed by pressing the precipitate between filter papers. About 130 g. of material should be obtained. Dissolve in water and dilute to 500 ml. This stock solution keeps indefinitely. Before use, a portion is diluted with 4 volumes of water. This diluted reagent is used in the procedure. The salt may also be prepared in quantity, preserved in the dry state, and dissolved in water as needed.

Standard Uric Acid Solution (*Benedict*). A stock solution is prepared as follows: Dissolve 9 g. of disodium hydrogen phosphate and 1 g. of sodium dihydrogen phosphate (pure crystalline salts) in about 200 to 300 ml. of hot water. If not perfectly clear, filter. Dilute the clear solution to 500 ml. with hot water and pour upon exactly 200 mg. of pure uric acid suspended in a few ml. of water in a liter volumetric flask. Mix until solution is complete. Cool, add exactly 1.4 ml. of glacial acetic acid, dilute to mark, and mix. Add 5 ml. of chloroform to prevent bacterial or mold growth. (5 ml. = 1 mg. of uric acid.) The *working standard* is prepared fresh weekly as follows: Transfer 10 ml. of stock solution to a 500 ml. volumetric flask, dilute to 400 ml.; add 3.5 ml. of concentrated hydrochloric acid, dilute to mark, and mix. (5 ml. = 0.02 mg. of uric acid.)

[64] A slight opalescence may be noted, due to a trace of colloidal silver chloride, but this disappears after the addition of the cyanide solution, and does not affect the accuracy of the results.

At 520 mμ, and in a 1-cm. cuvette, the standard described has a density of 0.180 (Fig. 150). Under these conditions, measurements are reliable up to 10 mg. per cent uric acid. For higher values, or with a deeper cuvette, use less filtrate plus water to 5 ml. in the acid silver chloride precipitation, and correct the calculations accordingly. The color in this procedure does not reach a constant value, changing gradually in intensity with time. This change takes place at about the same rate in the standard and in the unknowns. In serial analyses it is necessary, therefore, to reread the standard at suitable intervals and to calculate results in terms of the density of the standard at approximately the time the density of an unknown is determined.

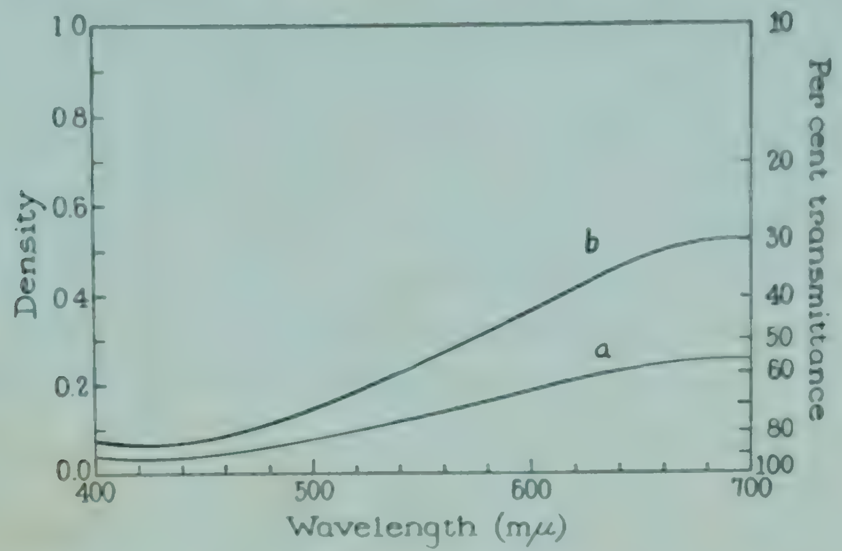

FIG. 150. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN NEWTON BLOOD URIC ACID METHOD.

For standards containing 0.01 mg. uric acid (*a*), and 0.02 mg. uric acid (*b*). Solution depth, 1 cm.

Interpretation. Results on human blood by this procedure are closely similar to those obtained by the previous method. Occurrence of turbidity is much less frequent than for other methods. For further interpretation see p. 562.

3. Method of Brown:[65] ***Principle.*** The tungstic acid filtrate is treated directly with a special uric acid reagent in the presence of optimal amounts of cyanide-urea solution. The color developed is compared with that of a uric acid standard treated similarly.

Procedure.[66] **Transfer 2 ml. of 1:10 tungstic acid filtrate to a test tube or cylinder graduated at 10 ml. In a similar tube place 2 ml. of standard uric acid solution containing 0.005 mg. of uric acid, and for photometric measurement prepare a third or "blank" tube containing 2 ml. of water. To each**

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[65] Brown: *J. Biol. Chem.*, **158**, 601 (1945).

[66] Reagents Required: *Sodium Cyanide Solution, 12 Per Cent.* Dissolve 12 g. of pure sodium cyanide in water in a beaker, transfer to a 100-ml. cylinder, add water to the mark, and mix with a stirring rod. *Handle this solution carefully, as it is extremely poisonous.* Keep in the refrigerator. It should be usable for about 2 weeks. Bring to room temperature before transferring a portion to a buret for daily use.

Urea Solution. Dissolve 50 g. of urea in sufficient water to make 100 ml. This solution keeps indefinitely at room temperature.

Uric Acid Reagent. Dissolve 100 g. of reagent-grade sodium tungstate and 20 g. of anhydrous disodium hydrogen phosphate in about 150 ml. of water in a flask with the aid of heat. Mix 25 ml. of concentrated sulfuric acid with about 75 ml. of water and pour the warm solution, slowly and with shaking, into the flask. Place a funnel in the mouth of the flask and in the funnel place a 200-ml. flask filled with ice water. Heat the mixture in the

tube add 2 ml. of cyanide solution *from a buret; this reagent is highly poisonous and must never be dispensed from a pipet.* Mix by lateral shaking, and add 2 ml. of urea solution to each tube. Again mix. Finally add 1 ml. of the uric acid reagent, mix by lateral shaking, and allow to stand at room temperature for 50 minutes. Dilute to the 10-ml. mark with water, stopper, and mix by inversion. Read in the colorimeter or photometer in the usual way. For photometric measurement, read at 520 mμ, setting the photometer to zero density with the blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.005 \times \frac{10}{2} \times 100 = \text{mg. uric acid per 100 ml. blood}$$

The standard described corresponds to a blood uric acid content of 2.5 mg. per cent. For values over 5 mg. per cent, repeat the analysis using 1 ml. of filtrate plus 1 ml. of water, and multiply the results by 2.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.005 \times \frac{10}{2} \times 100 = \text{mg. uric acid per 100 ml. blood}$$

At 520 mμ and in a 1-cm. cuvette, the density of the standard (corrected for the blank) is approximately 0.200. Satisfactory agreement with Beer's law is found up to blood concentrations of 6 mg. per cent. For higher values, or with deeper cuvettes, use 1 ml. of filtrate plus 1 ml. of water for the analysis, and multiply the results by 2.

Interpretation. By this method human whole blood shows a normal range of 2.2 to 3.5 mg. per cent for males and 1.9 to 2.9 mg. per cent for females. Results are lowered by only about 10 per cent or less when the uricase procedure (see p. 559) is applied to establish specificity. For further interpretation, see p. 562.

DETERMINATION OF AMINO ACIDS

Principle. The color developed by the reaction between amino acids and β-naphthoquinone-4-sulfonic acid in alkaline solution is the basis of this method. Originally proposed by Folin, the method was considerably improved by Danielson,[67] and this procedure is essentially the one described here, with heating to develop the color as suggested by Sahyun,[68] and with photometric measurement according to Frame, Russell, and Wilhelmi.[69]

Procedure.[70] **Prepare a tungstic acid filtrate of whole blood or plasma at a 1:10 dilution in the usual way (see p. 543). Transfer 5 ml. of protein-free**

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flask to boiling and boil gently for 1 hour, the funnel arrangement serving as a condenser. Cool and transfer to a 1-liter volumetric flask with rinsings, dilute to the mark with water, and mix.

Uric Acid Standard. Dilute 1 ml. of the Folin stock uric acid standard, containing 1 mg. of uric acid (see footnote 58) to 400 ml. with water and mix. This solution contains 0.005 mg. of uric acid in 2 ml., and keeps well for several days, particularly if kept cold.

[67] Danielson: *J. Biol. Chem.*, **101**, 505 (1933).

[68] Sahyun: *J. Lab. Clin. Med.*, **24**, 548 (1938–1939).

[69] Frame, Russell, and Wilhelmi: *J. Biol. Chem.*, **149**, 255 (1943). *See also* Russell: *J. Biol. Chem.*, **156**, 467 (1944), for a slightly modified procedure, claimed to give better results.

[70] Reagents Required: *Amino Acid Standard Solution.* A mixed standard containing glycine and glutamic acid is recommended for colorimetric measurement because of better match with blood filtrates. *Glycine Standard.* Dissolve exactly 0.268 g. of pure dry glycine in water and transfer with rinsings to a 500-ml. volumetric flask. Add 35 ml. of N hydro-

filtrate to a test tube graduated at 15 ml. In a similar tube place 5 ml. of amino acid standard solution, containing 0.03 mg. of amino acid nitrogen, and for photometric measurement prepare a blank tube containing 5 ml. of water.[71] Add 1 drop of 0.25 per cent alcoholic phenolphthalein solution to each tube, followed by 0.1 N sodium hydroxide solution drop by drop until a permanent pink color is obtained. Adjust by adding a little water where necessary so that all tubes are at approximately the same volume. To each tube add 1 ml. of borax solution, mix by tapping, and then add 1 ml. of freshly prepared naphthoquinone solution. Mix by tapping, and place the tubes immediately in a boiling water bath. Allow to remain 10 minutes, then remove and place in cold water for 5 minutes. To the cooled contents of each tube add 1 ml. of acid-formaldehyde solution, mix immediately, add 1 ml. of 0.1 N sodium thiosulfate solution, dilute immediately to the 15-ml. mark with water, and mix by inversion. Allow to stand for 10 to 30 minutes before reading in the colorimeter or photometer. For colorimetric measurement, match the unknown against the standard in the usual way. For photometric measurement, transfer the solutions to suitable containers and read in the photometer at 490 mμ, using the blank solution for setting the photometer to zero density.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.03 \times \frac{100}{0.5}$$
$$= \text{mg. amino acid nitrogen per 100 ml. blood or plasma}$$

If the unknown has an amino acid nitrogen content outside the range of 4 to 8 mg. per cent, repeat the determination using smaller portions of filtrate or standard as required, made up to 5 ml. with water, and correct the calculations accordingly.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.03 \times \frac{100}{0.5}$$
$$= \text{mg. amino acid nitrogen per 100 ml. blood or plasma}$$

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chloric acid and 1 g. of sodium benzoate. Add water to dissolve and dilute to 500 ml. with water. Mix. *Glutamic Acid Standard.* Dissolve exactly 0.525 g. of pure dry glutamic acid in water, transfer to a 500-ml. volumetric flask, add 35 ml. of N hydrochloric acid and 1 g. of sodium benzoate as above, dilute to 500 ml. with water, and mix. Each of these stock standards contains 0.1 mg. of amino acid *nitrogen* per ml. They are stable indefinitely. To prepare the *mixed standard,* transfer 3 ml. of both the glycine and glutamic acid standards to a 100-ml. volumetric flask, dilute to the mark with water, and mix. This standard contains 0.03 mg. of amino acid nitrogen in 5 ml. and is usable for one week if kept in the cold.

0.25 Per Cent Alcoholic Phenolphthalein. Dissolve 0.25 g. of phenolphthalein in 95 per cent alcohol and dilute to 100 ml.

0.1 N Sodium Hydroxide. Dilute 10 ml. of N sodium hydroxide solution to 100 ml. with water and mix.

Borax Solution. Dissolve 15 g. of borax (sodium tetraborate, $Na_2B_4O_7 \cdot 10H_2O$) in water and dilute to 1 liter. Stable indefinitely.

Naphthoquinone Solution. Dissolve 0.25 g. of β-naphthoquinone-4-sulfonic acid in water and dilute to 50 ml. Prepare immediately before using, and discard remaining solution.

Acid-Formaldehyde Solution. Dilute 11.3 ml. of 40 per cent formaldehyde to 1 liter with water. Mix 4 volumes of this solution with 3 volumes of 1.5 N hydrochloric acid and 1 volume of glacial acetic acid. Stable indefinitely.

0.1 N Sodium Thiosulfate Solution. This need not be standardized. Dissolve 25 g. of crystalline sodium thiosulfate in water, dilute to 1 liter with water, and mix. Usable indefinitely.

[71] A water blank is valid only if the reagents used in preparing the blood filtrate (tungstate, acid) are substantially ammonia-free.

For the spectrophotometric characteristics of the color obtained in this method, see Frame, Russell, and Wilhelmi (*loc. cit.*). At 490 mμ and in a 1-cm. cuvette, the density of the standard is approximately 0.400; at 520 mμ, which may be used with slightly less accuracy, the density is about 25 per cent less. Up to about 15 mg. per cent amino acid nitrogen may therefore be determined accurately under these conditions; for higher levels, repeat the analysis with a smaller portion of filtrate.

Interpretation. The amino acid nitrogen content of whole blood as determined by this method ranges from 5 to 8 mg. per cent. A slight increase is noted after the ingestion of protein foods but no significant change is found in fasting. High values have been observed in uremic nephritis although a remarkable constancy is observed in most pathological conditions. Increases have, however, been noted in leukemia and particularly in acute yellow atrophy of the liver. Insulin markedly reduces the amino acid content of blood. Plasma averages around 4 to 5 mg. per cent, with serum slightly higher: according to MacFadyen,[72] this increase is due to release of amino acids during clotting. The concentration of amino nitrogen in corpuscles is about twice that of whole blood. Of naturally occurring interfering substances, glutathione is of chief significance; since this substance is present largely in the corpuscles, interference from this source is minimized if plasma (or an "unlaked" blood filtrate[67]) is taken for analysis. Uric acid at a concentration of 1 mg. per cent gives color equivalent to 0.1 mg. per cent amino acid nitrogen;[69] correction may be necessary on bloods with high uric acid content. The sulfonamides also interfere if present; a free sulfonamide level of 10 mg. per cent being equivalent to 0.8 mg. per cent amino acid nitrogen;[69] correction may also be applied here. Despite these limitations, the method is considered to be reliable and satisfactory for blood analysis.

Other Methods. Other colorimetric methods are not as satisfactory as the one described here. Gasometric methods include the classical nitrous acid method of Van Slyke,[73] which is based upon the measurement of nitrogen liberated from α-amino acids on treatment with nitrous acid; and the gasometric ninhydrin method of Van Slyke and Dillon,[74] in which the carbon dioxide liberated from the carboxyl group of α-amino acids on boiling with ninhydrin is measured. For description of the ninhydrin method as applied to the determination of the amino acid content of urine, see Chapter 31. Microbiological methods for the determination of individual amino acids are described in Chapter 33.

DETERMINATION OF GLUCOSE

Introduction. The majority of methods for the determination of blood glucose are based upon the ability of glucose in hot alkaline solution to reduce certain metallic ions, of which the cupric and ferricyanide ions are most commonly used. The extent of reduction is then established by colorimetric, titrimetric, or gasometric methods. Methods involving or including yeast fermentation,[75] while undoubtedly the most specific for glucose, are not used routinely. It has long been known that there are reducing substances other than glucose present in blood, and that these may occur in sufficient amount to increase considerably the "apparent" glucose value. The term "saccharoid" has been proposed to designate the non-glucose reducing fraction of blood.[76] The various blood sugar methods differ in their specificity for glucose, and therefore tend to give slightly or even significantly different values for both normal and pathological blood. In using a method, or in interpreting results obtained by it, particularly in the early literature, it is important to know the relationship between the values obtained by the particular method and the "true glucose" values, as well as the values obtained by other and possibly more specific methods. It is also important to know whether the method employs venous or arterial (capillary) blood, since this may influence the interpretation of results (see p. 577). Because of the free diffusibility of glucose between red cells and plasma, distinction between the analysis of whole blood and plasma is relatively unimportant except for methods which include the reduction due to saccharoids, which are found chiefly in the red cells.

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[72] MacFadyen: *J. Biol. Chem.*, **145**, 387 (1942).

[73] See Van Slyke and Peters: *Quantitative Clinical Chemistry*, Vol. II (Methods), Baltimore, Williams and Wilkins Co., 1932.

[74] Van Slyke and Dillon: *Proc. Soc. Exptl. Biol. Med.*, **34**, 362 (1936); Van Slyke, Dillon. MacFadyen, and Hamilton: *J. Biol. Chem.*, **141**, 627 (1941). MacFadyen: *loc. cit.*

1. *Method of Folin and Wu:*[77] *Principle.* The protein-free blood filtrate is heated with alkaline copper solution, using a special tube to prevent reoxidation. The cuprous oxide formed is treated with a phosphomolybdic acid solution, a blue color being obtained which is compared with that of a standard.

***Procedure.*[78] Transfer 2 ml. of the tungstic acid blood filtrate (or 1 ml. plus 1 ml. of water if very high blood sugar values are expected) to a Folin-Wu**

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[75] See Somogyi: *J. Biol. Chem.*, **78**, 117 (1928); Van Slyke and Hawkins: *ibid.*, **83**, 51 (1929); Holden: *ibid.*, **119**, 347 (1937); Winzler: *Science*, **99**, 327 (1944).

[76] Benedict: *J. Biol. Chem.*, **92**, 141 (1931). According to Fashena (*J. Biol. Chem.*, **100**, 357 (1933); Fashena and Stiff: *ibid.*, **137**, 21 (1941)) the saccharoid fraction of normal human blood is accounted for almost entirely by glutathione and glucuronic acid.

[77] Folin and Wu: *J. Biol. Chem.*, **41**, 367 (1920). Folin (*J. Biol. Chem.*, **67**, 357 (1926); **82**, 83 (1929)) has critically examined and improved this method to obtain a more accurate measure of true sugar and better proportionality between sugar concentration and color intensity. The original Folin-Wu method is here described because it continues to have wide usage, especially in hospital laboratories.

[78] Reagents Required: *Standard Sugar Solutions.* Three standard sugar solutions should be on hand: (*a*) a stock solution, 1 per cent glucose made up in saturated benzoic acid solution; (*b*) a solution containing 2 mg. of sugar in 1 ml. (20 ml. of stock solution diluted to 100 ml. with water); (*c*) solutions containing 0.2 and 0.4 mg. of sugar in 2 ml., made by dilution of (*b*) with water. The dilute standards are best made up fresh a couple of times a week. Merck's *highest purity* dextrose is satisfactory.

Alkaline Copper Solution. Dissolve 40 g. of pure anhydrous sodium carbonate in about 400 ml. of water and transfer to a liter flask. Add 7.5 g. of tartaric acid, and when the latter has dissolved add 4.5 g. of crystallized copper sulfate. Mix and make up to a volume of 1 liter. If the chemicals used are not pure a sediment of cuprous oxide may form in the course of one or two weeks. If this should happen, remove the clear supernatant reagent with a siphon, or filter through a good quality filter paper. The reagent seems to keep indefinitely. To test for the absence of cuprous copper in the solution, transfer 2 ml. to a test tube and add 2 ml. of the molybdate phosphate solution; the deep blue color of the copper should almost completely vanish. In order to forestall improper use of this reagent attention should be called to the fact that it contains extremely little alkali, 2 ml. by titration (using the fading of the blue copper tartrate color as indicator) requiring only about 1.4 ml. of normal acid.

Phosphomolybdic Acid Solution. To 35 g. of molybdic acid and 5 g. of sodium tungstate, add 200 ml. of 10 per cent sodium hydroxide and 200 ml. of water. Boil vigorously for 20

sugar tube graduated at 25 ml. (Fig. 151) and to other similar tubes add 2 ml. of standard sugar solutions containing 0.2 and 0.4 mg. respectively of glucose. To each tube add 2 ml. of the alkaline copper solution. The surface of the mixtures must now have reached the constricted part of the tube. Transfer the tubes to a rapidly boiling water bath and heat for 8 minutes. Cool in running water without shaking. To each tube add 2 ml. of phosphomolybdic acid reagent. After about 1 minute dilute to the mark with water and mix. It is essential that adequate attention be given to this mixing because the greater part of the blue color is formed in the bulb of the tube. Compare in a colorimeter using the standard which most nearly matches the unknown. For photometric measurement, transfer the solutions to suitable containers and determine the densities at 420 mμ, setting the photometer to zero density with a blank obtained by treating 2 ml. of water with alkaline copper reagent, heating, etc., just as in the analysis of the blood filtrate.

CALCULATION. *For colorimetric measurement,* use the standard which most closely matches the unknown, and calculate as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. glucose in standard} \times \frac{100}{0.2}$$
$$= \text{mg. glucose per 100 ml. blood}$$

Unless the readings of the standard and unknown are within a few millimeters of each other, results obtained by this calculation are somewhat incorrect because the color is not strictly proportional to the concentration of glucose. Correction of observed blood sugar values[79] may be of importance when the values are near the critical levels corresponding to hypoglycemia and to the threshold range. Instead of applying corrections it may be advisable to employ, in place of the usual standards, standards containing 0.1 and 0.3 mg. of glucose in 2 ml. (corresponding to blood sugar values of 50 and 150 mg. per 100 ml., respectively) or to dilute to an approximate color match (using the graduated sugar tubes of Rothberg and Evans)[80] prior to matching in the colorimeter.

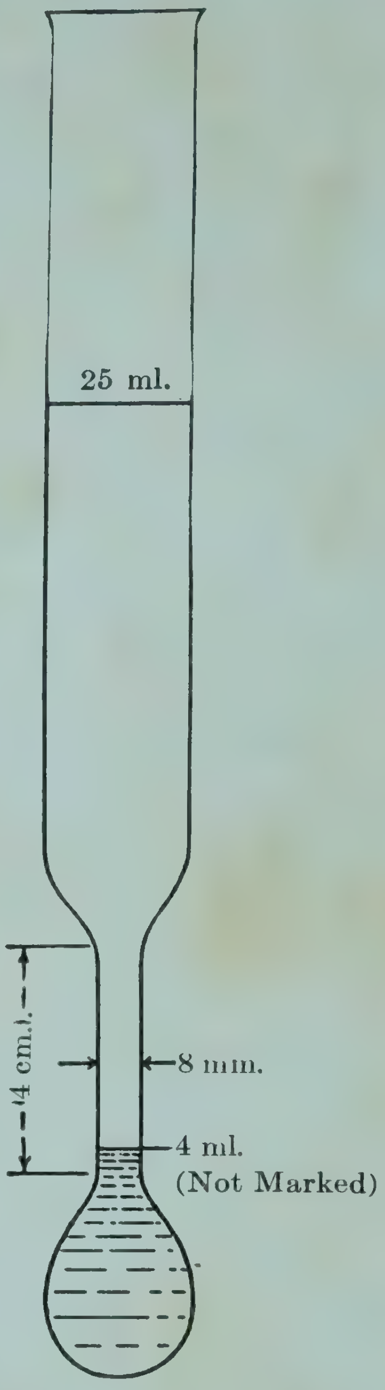

FIG. 151. FOLIN-WU SUGAR TUBE.

For photometric measurement, only one standard and a blank are required, and the calculation is as follows:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. glucose in standard} \times \frac{100}{0.2}$$
$$= \text{mg. glucose per 100 ml. blood}$$

At 420 mμ and in a 1-cm. cuvette, the density of the standard containing 0.4 mg. of glucose, corresponding to 200 mg. per cent of blood glucose, is approximately 0.300 (Fig. 152). Satisfactory agreement with Beer's law is found under these conditions up

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to 40 minutes so as to remove nearly the whole of the ammonia present in the molybdic acid. Cool, dilute to about 350 ml. and add 125 ml. of concentrated (85 per cent) phosphoric acid. Dilute to 500 ml.

[79] Oser and Karr (*J. Biol. Chem.*, **67**, 319 (1926)) have published tables and curves correcting for the deviation from Beer's law.

[80] Rothberg and Evans: *J. Biol. Chem.*, **58**, 443 (1923).

to about 400 mg. per cent blood glucose. For higher values, or with deeper cuvettes, carry out the analysis using less filtrate plus water to 2 ml., and correct the calculations accordingly.

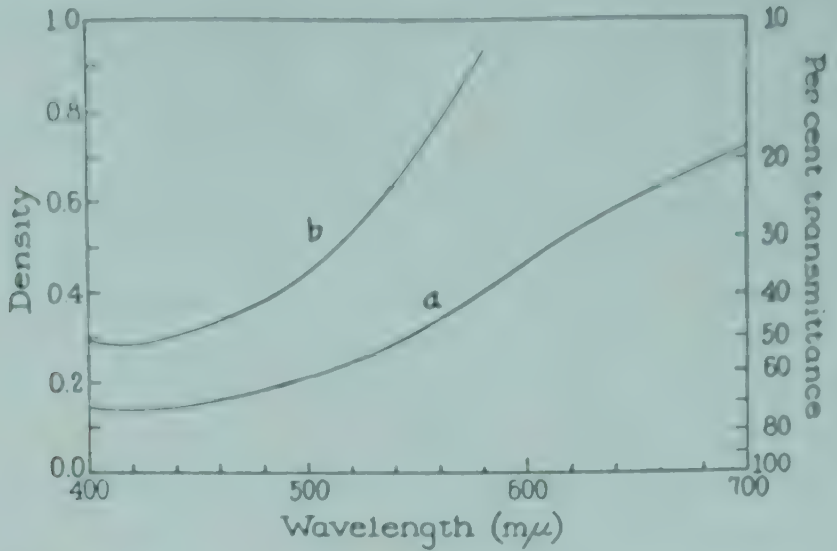

FIG. 152. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN FOLIN-WU BLOOD SUGAR METHOD.

For standards containing (*a*) 0.2 mg. and (*b*) 0.4 mg. glucose. Solution depth, 1 cm.

Interpretation. The normal range of fasting venous blood sugar values by this method is 90 to 120 mg. per 100 ml. of whole blood. This range is undoubtedly "enhanced" to the extent of possibly 20 to 30 mg. per cent by the effect of non-glucose reducing substances (saccharoids), but the saccharoid fraction appears to be relatively constant and therefore its presence does not influence unduly the interpretation of variations in blood sugar level as obtained by this procedure. Most of the early data in the American literature on blood sugar content were obtained by this method, but it is gradually being superseded by the more specific methods described subsequently.

In mild diabetes values of 140 to 300 mg. per cent are obtained, and in severe diabetes values up to 1200 mg. per cent have been noted. Hyperglycemia is also observed in severe nephritis, pancreatic disease, hyperthyroidism, and certain hepatic disorders. Experimentally, the administration of adrenaline and the induction of ether anesthesia lead to elevated blood sugar values. Low blood sugar values are found after the administration of insulin, in such hypoendocrine disturbances as Addison's disease, hypopituitarism, cretinism, and myxedema, and in the clinical condition known as hyperinsulinism. For carbohydrate tolerance test, see p. 579.

2. *Method of Benedict:*[81] *Principle.* The protein-free filtrate is heated with an alkaline copper reagent containing tartrate, alanine, and bisulfite. The reduced copper is determined colorimetrically after treatment with phosphomolybdic acid. The alkaline copper reagent used here is practically unaffected by the nonsugar reducing materials present in blood, hence the method gives values distinctly lower than with the Folin-Wu method, and presumably nearer the true glucose content. The reagent is also adapted to the determination of the nonsugar reducing value of blood (see original paper).

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[81] Benedict: *J. Biol. Chem.*, **92**, 141 (1931).

Procedure.[82] **Place 2 ml. of 1:10 protein-free filtrate[83] in a Folin-Wu sugar tube, and in a second similar tube place 2 ml. of standard glucose solution. To each tube add 2 ml. of the copper reagent containing bisulfite. Mix by lateral shaking, and place in a vigorously boiling water bath for 6 minutes. Cool by placing in cold water for 1 to 2 minutes (avoid shaking at this stage). To each tube add 2 ml. of color reagent, mix by vigorous lateral shaking, and after about 1 minute dilute with water to the 25-ml. mark. Mix the contents thoroughly by repeated inversion, allow to stand at least 10 minutes, and read in the colorimeter or photometer within the next 30 minutes or so. For colorimetric measurement, compare against the standard in the usual way. For photometric measurement, transfer portions of the solutions to suitable containers and determine the densities in the photometer under the same conditions as described for the Folin-Wu method, p. 569.**

CALCULATION. Same as for Folin-Wu method.

This procedure gives better proportionality between color and concentration than the Folin-Wu method. A standard glucose solution containing 0.2 mg. of glucose, equivalent to 100 mg. per cent blood sugar, is therefore suitable in colorimetric comparison for values up to 200 mg. per cent blood sugar. For higher levels, use a stronger standard or less filtrate plus water to 2 ml. In photometric measurement, agreement with Beer's law is excellent up to 500 mg. per cent blood sugar, at 420 mμ, and in a 1-cm. cuvette. For higher values, or with a deeper cuvette, carry out the analysis using less filtrate plus water to 2 ml., and correct the calculations accordingly.

Interpretation. Normal values on whole blood by this method range from 70 to 100 mg. per cent blood glucose. The interpretation of variation from the normal range is similar to that already presented in connection with the Folin-Wu method.

3. Somogyi-Shaffer-Hartmann Method:[84] ***Principle.*** Hemolyzed blood is deproteinized with zinc hydroxide, giving a filtrate containing practically no reducing substances other than sugar. The sugar is estimated by iodometric titration of reduced copper. Precipitation of the protein with copper salts is just as satisfactory in the case of whole blood and is better for plasma or serum.[85]

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[82] Reagents Required: *Copper Reagent.* Dissolve 15 g. of anhydrous sodium carbonate, 3 g. of alanine, and 2 g. of Rochelle salt in about 250 ml. of distilled water. Dissolve 3 g. of crystalline copper sulfate in about 100 ml. of distilled water and add this solution with stirring to the carbonate-alanine-tartrate solution. Dilute to 500 ml. and mix. Keep in a cool place. This reagent will keep ready mixed from 4 to 6 weeks. If, after several weeks, there may be a slight growth of mold in the solution, it may be removed at any time by pouring the solution through a loose plug of absorbent cotton in a funnel, leaving the efficiency of the reagent unaffected.

1 Per Cent Sodium Bisulfite Solution. Kept in a 100-ml. dropping bottle. The solution should be prepared fresh about once a month.

Copper Reagent Containing Bisulfite. Measure into a cylinder a volume of the copper reagent which will be used up in one or two days, and add 1 drop of the bisulfite solution for each ml. of copper reagent, or 1 ml. of bisulfite for each 20 ml. of reagent. Mix. Do not use after the second day.

Color Reagent. To 150 g. of pure molybdic acid and 75 g. of anhydrous Na_2CO_3 in a large Erlenmeyer flask, add 500 ml. of water in small portions while shaking. Heat to boiling. Filter. Wash residue on filter with hot water until filtrate plus washings equal 600 ml. Add 300 ml. of 85 per cent H_3PO_4, cool, and dilute to 1 liter.

Standard Glucose Solution. See footnote 78, p. 568.

[83] Benedict uses a tungstomolybdate filtrate (Benedict: *J. Biol. Chem.*, **92**, 135 (1931)). A Folin-Wu filtrate may also be used.

[84] Somogyi: *J. Biol. Chem.*, **86**, 655 (1930); **70**, 599 (1926). Urea, creatinine, and creatine may also be determined in the zinc filtrate but only traces of uric acid are found. Lower but significant values for nonprotein nitrogen may be obtained (*J. Biol. Chem.*, **87**, 339 (1930)). For an improved procedure, suitable for colorimetric as well as titrimetric determination, see Somogyi: *J. Biol. Chem.*, **160**, 61, 69 (1945).

Procedure. (a) DEPROTEINIZATION. **Take 1 volume of blood with 7 volumes of water. Add 1 volume of 10 per cent solution of $ZnSO_4{\cdot}7H_2O$. Mix. Add with continuous shaking 1 volume of 0.5 N NaOH. Stopper the flask, shake well, and filter after a few minutes through dry filter paper. For accurate work measure the blood with an Ostwald pipet calibrated "to contain" and rinse with the laking water. For serum or plasma dilute with 8 volumes of water and add 0.5 volume of each of the reagents. Another procedure for whole blood is to add 8 volumes of an acid zinc solution (12.5 g. of $ZnSO_4{\cdot}7H_2O$ and 125 ml. of 0.25 N H_2SO_4 with water to make 1 liter) and 1 volume of 0.75 N NaOH. Shake vigorously and filter after a few minutes. 50 ml. of the acid zinc solution should require 6.7 to 6.8 ml. of 0.75 N NaOH to give a permanent pink with phenolphthalein. Determine sugar as below. For microtechnique introduce into a test tube or 25-ml. Erlenmeyer flask 5.8 ml. of water, add 0.2 ml. of blood from an accurate capillary pipet, rinsing several times with the laking water. Mix. Add 1 ml. of 1.8 per cent $ZnSO_4{\cdot}7H_2O$. Mix. Add with continuous shaking 1 ml. of 0.1 N NaOH.[86] Stopper, shake well, and filter after a few minutes through a dry thin paper (Schleicher and Schüll No. 597, 70 mm.). Use 5 ml. of filtrate equivalent to 0.125 ml. of blood. Determine sugar by procedure described.**

(b) DETERMINATION OF SUGAR. **Measure 5 ml. of the copper reagent[87] into a large test tube (25 × 250 mm.) and add 5 ml. of the sugar solution[88] containing not less than 0.1 nor more than 2.0 mg. of glucose. Shake gently to mix, cover the tube with a small funnel, bottle cap, or glass bulb, and keep in a boiling water bath for 15 minutes. Cool (avoid shaking) by placing in a shallow dish of water until the temperature falls to 35° to 40° C. (not below 30°). Add 1 ml. of 5 N H_2SO_4 (or equivalent amount) and see that all Cu_2O is promptly dissolved. Avoid reoxidation of reduced copper or loss of iodine by too vigorous agitation. After about 2 minutes titrate with 0.005 N sodium thiosulfate, using starch as an indicator toward the end of the titration. Run a blank on 5 ml. of reagent after heating with an equal volume of water.**

CALCULATION. From the blank titration subtract the titration of the unknown. This gives ml. of thiosulfate required. For the glucose equivalent consult the following table, which applies to the usual 1:10 dilutions of blood. For other cases the actual amount of glucose in the 5 ml. of solution used for the determination is obtained by dividing the value in the table by 200.

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[85] Somogyi: *J. Biol. Chem.*, **90**, 725 (1931). Lake 1 volume of blood in 7 volumes of water. Add 1 volume of 7 per cent $CuSO_4{\cdot}5H_2O$ and mix. Then add, with continuous shaking, 1 volume of 10 per cent $Na_2WO_4{\cdot}2H_2O$. Stopper the flask. Shake well. Filter through dry filter paper after a few minutes. For plasma or serum use the same procedure but use 5 per cent copper sulfate and 6 per cent sodium tungstate solutions.

[86] 10 ml. of the zinc sulfate solution diluted with 60 ml. of water and slowly titrated with the NaOH should require 12 to 12.2 ml. for a permanent pink with phenolphthalein.

[87] *Copper Reagent.* Dissolve 12 g. of Rochelle salt, 20 g. of sodium carbonate (anhydrous), and 25 g. of sodium bicarbonate in about 500 ml. of water. Into this pour with stirring 6.5 g. of copper sulfate (crystalline) dissolved in about 100 ml. of water. Dissolve 10 g. of potassium iodide, 0.80 g. (weighed to cg.) of potassium iodate, and 18 g. of potassium oxalate in about 200 ml. of water. Add to the main solution and dilute to 1 liter.

Thiosulfate. For preparation of 0.1 N solution see Appendix. Dilute this every day or two as needed to 0.005 N.

[88] Strongly acid sugar solutions must be neutralized to phenol red. For above filtrates or tungstic acid filtrates this is not necessary.

AMOUNTS OF GLUCOSE CORRESPONDING TO TITRATION VALUES WHEN 5 ML. 1:10 BLOOD FILTRATE AND 5 ML. COPPER REAGENT (MODIFIED) ARE HEATED IN WATER BATH FOR 15 MINUTES

Milliliters of 0.005 N Thiosulfate	*Tenths of 1 ml. of 0.005 N Sodium Thiosulfate*									
	0	1	2	3	4	5	6	7	8	9
	Mg. Glucose in 100 ml. Blood									
0	..	..	21	23	26	29	31	34	36	39
1	41	44	46	49	51	53	56	58	61	63
2	65	68	70	72	75	77	80	82	84	86
3	89	92	94	97	99	101	103	106	108	110
4	113	115	117	119	121	124	126	128	130	132
5	135	137	139	141	143	146	148	150	152	154
6	157	159	161	163	165	168	170	172	174	176
7	179	181	183	185	187	190	192	194	196	199
8	201	203	205	207	210	212	214	216	218	221
9	223	225	227	230	232	234	237	239	241	243
10	245	248	250	252	254	256	259	261	263	265
11	267	270	272	274	276	279	281	283	285	288
12	290	292	294	296	299	301	303	305	308	310
13	312	314	316	318	321	323	326	328	330	332
14	334	337	339	341	343	345	347	350	352	354
15	356	359	361	363	365	367	370	372	374	376
16	378	381	383	386	388	390	392	394	396	398
17	400	..	..	..	..	..	..	..	..	..

Interpretation. Normal values by this method range from 70 to 100 mg. per cent and are presumably very close to the true glucose values. The specificity for glucose appears to reside in the method of deproteinization, since filtrates prepared as described may be analyzed for glucose by the Folin-Wu or Benedict colorimetric methods to give results substantially identical with those obtained by the Shaffer-Hartmann iodometric titration. For the principle of the Shaffer-Hartmann procedure, see Chapter 31.

4. Nelson-Somogyi Method:[89] ***Principle.*** Blood is deproteinized by a zinc hydroxide–barium sulfate procedure which gives a filtrate containing practically no reducing substances other than glucose. The zinc-barium filtrate is heated with an alkaline copper reagent and then treated with a special arsenomolybdate color reagent. The color developed is compared with that obtained from a known amount of glucose.

Procedure.[90] (a) DEPROTEINIZATION. **Place 1 ml. of blood in a 50-ml. flask. Add 9.5 ml. of barium hydroxide solution, mixing by rotation. Add 9.5 ml. of**

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[89] Nelson: *J. Biol. Chem.*, **153**, 375 (1944); Somogyi: *J. Biol. Chem.*, **160**, 62 (1945). Adapted by permission from the official procedures of the American Association of Clinical Chemists.

[90] Reagents Required: *Barium Hydroxide Solution.* Dissolve 90 g. of $Ba(OH)_2 \cdot 8H_2O$ in distilled water and dilute to 2000 ml. in a graduated cylinder. Filter if cloudy. Store in well-

zinc sulfate solution, mixing by rotation. Shake vigorously and filter on a dry filter paper, collecting the filtrate in a dry flask.

(b) DETERMINATION OF GLUCOSE. **Measure 0.5 ml. of the barium-zinc filtrate into a test tube calibrated at 10 ml. Add 1 ml. of alkaline copper reagent, mix by tapping, cover the top of the tube with a marble, and place upright in a boiling water bath for 20 minutes. Cool by placing the tube in water at room temperature for 1 minute. Add 1 ml. of arsenomolybdate color reagent, mixing by tapping; then dilute to 10 ml. with distilled water. Mix by inversion. Compare in a colorimeter against a simultaneously prepared standard obtained by treating 0.5 ml. of standard glucose solution (see footnote 90) exactly as described for the blood filtrate. For photometric measurement, prepare a blank by carrying out the procedure as described on 0.5 ml. of water. Set the photometer to zero density with the blank, and measure the optical densities of standard and unknown at 540 mμ.**

CALCULATION. *For colorimetric measurement,* calculate as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. glucose in standard} \times \frac{100}{0.025}$$
$$= \text{mg. glucose per 100 ml. blood}$$

It is convenient to set up three standards, covering low, normal, and high values of blood glucose, as follows: I, containing 0.0125 mg. glucose, for low values; II, containing 0.025 mg. glucose, for normal values; III, containing 0.050 mg. glucose, for high values. The unknown is then compared against the standard it most closely

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stoppered containers filled to capacity.

Zinc Sulfate Solution. Dissolve 100 g. of $ZnSO_4 \cdot 7H_2O$ in distilled water, dilute to 2000 ml. in a graduated cylinder, and mix. Stable indefinitely.

It is most important that these two solutions exactly neutralize each other. Check by measuring 10 ml. of zinc sulfate solution into a flask, diluting with about 50 ml. of distilled water, add 4 drops of phenolphthalein solution, and titrate with the barium hydroxide solution, slowly and with constant shaking, until 1 drop of alkali turns the mixture pink. If it requires more or less than 10.00 ml. ± 0.05 ml. of barium hydroxide, dilute one or the other solution as required and check again. Continue until the two solutions are exactly equivalent. Protect the barium hydroxide solution in the dispensing bottle with a soda-lime tube in the stopper, and test both solutions occasionally by making a trial filtrate on blood. Satisfactory solutions will give a clear filtrate which filters rapidly and shows little tendency to foam.

Alkaline Copper Reagent: Solution A. Dissolve 50 g. of anhydrous sodium carbonate, 50 g. of Rochelle salt, 40 g. of sodium bicarbonate, and 400 g. of anhydrous sodium sulfate in about 1600 ml. of distilled water, and dilute to 2 liters. Mix and filter if not clear. Store at room temperature. If a sediment forms in a few days, filter again. *Solution B.* Dissolve 150 g. of $CuSO_4 \cdot 5H_2O$ in distilled water and dilute to 1 liter. Add 0.5 ml. of concentrated sulfuric acid, and mix. *Alkaline Copper Reagent.* On the day it is to be used, place 4 ml. of Solution B in a 100-ml. graduated cylinder, dilute to 100 ml. with Solution A, and mix.

Arsenomolybdate Color Reagent. Dissolve 100 g. of ammonium molybdate in 1800 ml. of distilled water. Add 84 ml. of concentrated sulfuric acid, with stirring. Dissolve 12 g. of disodium orthoarsenate ($Na_2HAsO_4 \cdot 7H_2O$) in 100 ml. of distilled water, and add it with stirring to the acidified molybdate solution. Place the mixture in an incubator at 37° C. for 1 to 2 days. Store in a glass-stoppered brown glass bottle. This solution should be stable indefinitely.

Standard Glucose Solutions. Prepare a stock standard by dissolving exactly 1.00 g. of highest purity anhydrous glucose in about 10 to 15 ml. of 0.2 per cent benzoic acid solution, transferring quantitatively to a 100-ml. volumetric flask, diluting to the mark with the benzoic acid solution, and mixing. This solution is stable indefinitely, and contains 10 mg. of glucose per ml. Standard I (0.0125 mg. glucose per 0.5 ml.) is prepared by diluting 0.5 ml. of the stock standard to 200 ml. with 0.2 per cent benzoic acid solution and mixing. For Standard II (0.025 mg. glucose per 0.5 ml.) dilute 1.0 ml. of stock standard to 200 ml. as described for Standard I, and for Standard III (0.050 mg. glucose per 0.5 ml.) dilute 2.0 ml. of stock standard to 200 ml. These dilute solutions in 0.2 per cent benzoic acid solution keep indefinitely at room temperature.

matches. The calculation can be simplified as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times f = \text{mg. glucose per 100 ml. blood}$$

where f is a factor with the following values: for standard I, 50; for standard II, 100; for standard III, 200.

For photometric measurement, the calculation is as follows:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. glucose in standard} \times \frac{100}{0.025}$$
$$= \text{mg. glucose per 100 ml. blood}$$

A standard containing 0.025 mg. of glucose is satisfactory for blood samples containing up to 350 mg. per cent blood sugar. With this standard the calculation simplifies to:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 100 = \text{mg. glucose per 100 ml. blood}$$

For samples containing more than 350 mg. per cent blood sugar, repeat the analysis using a smaller aliquot of filtrate plus water to make 0.5 ml., and correct the calculations accordingly. The colors obtained by this method are said to be stable for more than 24 hours.

Interpretation. Normal values on fasting venous blood by this method range between 65 and 110 mg. per 100 ml. of whole blood, with an average value of 85 mg. per 100 ml. The interpretation of variation from the normal range is similar to that already presented in connection with the Folin-Wu method.

5. *Micromethod for Glucose in 0.1 Ml. of Blood* (*Folin and Malmros*):[91]

Principle. The sugar is oxidized with alkaline potassium ferricyanide, and the ferrocyanide produced is measured colorimetrically or photometrically after conversion to Prussian blue.

Procedure. **With an accurate 0.1-ml. pipet[92] collect 0.1 ml. of blood and transfer it to 10 ml. of dilute tungstic acid[93] in a centrifuge tube. Stir well and**

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[91] Folin and Malmros: *J. Biol. Chem.*, **83**, 115 (1929); Folin: *J. Biol. Chem.*, **77**, 421 (1928). See also Horvath and Knehr: *J. Biol. Chem.*, **140**, 869 (1941). For a method based upon titration of the ferrocyanide formed using ceric sulfate, see Miller and Van Slyke: *J. Biol. Chem.*, **114**, 583 (1936); MacFadyen and Van Slyke: *ibid.*, **149**, 527 (1943). See also the Hawkins and Van Slyke method, Chapter 31.

[92] A capillary pipet about 7 cm. in length per 0.1 ml., calibrated "to contain," using mercury (0.1 ml. weighs 1.355 g.). Sold by dealers in laboratory supplies.

[93] *Dilute Tungstic Acid Solution.* Transfer 20 ml. of 10 per cent sodium tungstate to a liter volumetric flask. Dilute to about 800 ml. Add with shaking 20 ml. of ⅔ N sulfuric acid and dilute to volume.

Potassium Ferricyanide Solution. Dissolve 2 g. of c.p. potassium ferricyanide in distilled water and dilute to a volume of 500 ml. Keep the major part of the solution in a brown bottle in a dark closet. Keep the reagent in daily use also in a brown bottle.

Sodium Cyanide-Carbonate Solution. Transfer 8 g. of anhydrous sodium carbonate to a 500 ml. volumetric flask. Add 40 to 50 ml. of water and shake to promote rapid solution. With a cylinder, add 150 ml. of freshly prepared 1 per cent sodium cyanide solution, dilute to volume, and mix.

Ferric Iron Solution. Fill a liter cylinder with water. Suspend on a copper wire screen, just below the surface, 20 g. of soluble gum ghatti, and leave overnight (18 hours). Remove the screen, and strain the liquid through a double layer of a clean towel. Add to this extract a solution of 5 g. of anhydrous ferric sulfate in 75 ml. of 85 per cent phosphoric acid plus 100 ml. of water. Add to the mixture, a little at a time, about 15 ml. of 1 per cent potassium permanganate solution to destroy certain reducing materials present in gum ghatti. The centrifuge. Transfer 4 ml. of the water-clear supernatant fluid to a test tube graduated (with a ring going all around) at 25 ml. Transfer 4 ml. of the standard sugar solution to another similar tube. To each tube add 2 ml. of the 0.4 per cent potassium ferricyanide solution and 1 ml. of the cyanide-carbonate solution. Heat immediately in boiling water for 8 minutes[94] and cool in running water for 1 to 2 minutes. Add 5 ml. of the ferric iron solution and mix. Let stand for 1 to 2 minutes and then dilute with water nearly, but not quite, to the 25-ml. mark. Add 2 drops of alcohol to cut the foam and dilute to the mark. Mix. Allow to stand 10 minutes and read within the next 30 minutes.

For colorimetric comparison, half fill the colorimeter cups with the green-colored standard, set the two plungers at a height of 20 mm., and cover the opening of the light box with the picric acid light filter.[95] Adjust the position of the colorimeter and of the mirror glass reflector until the two fields look exactly alike. The adjustment is easier if the colorimeter is kept on a piece of plate glass polished on one side and rough on the other. Rinse one colorimeter cup and plunger with the unknown solution and pour the unknown into the cup to a suitable height. Compare in the usual way.

For photometric measurement, transfer the solutions to suitable containers and determine the densities in a photometer at 520 mμ. Set the photometer to zero density with water alone, rather than use a blank (see below).

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.04 \times \frac{10}{4.0} \times \frac{100}{0.1} = \text{mg. glucose per 100 ml. blood}$$

The proportionality between color and concentration is excellent in this method, so that readings between 5 and 40 mm. may be accepted, with the standard at 20 mm., if perfect equality as to light and color is obtained. If the blood glucose concentration is over 400 mg. per cent, repeat with 2 ml. of filtrate plus 2 ml. of water, and multiply the results by 2.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.04 \times \frac{10}{4.0} \times \frac{100}{0.1} = \text{mg. glucose per 100 ml. blood}$$

At the wavelength specified, and in a 1-cm. cuvette, the standard corresponding to

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slight turbidity of the solution will disappear completely, if kept at 37° C. for a few days. The use of Duponol, a synthetic detergent, has been recommended in place of gum ghatti (Klendshoj and Hubbard: *J. Lab. Clin. Med.*, **25**, 1102 (1939–1940); Horvath and Knehr: *loc. cit.*).

Standard Glucose Solution. The working standard contains 0.01 mg. of glucose per ml. (see footnote 78, p. 568).

[94] Heating for 20 minutes instead of 8 minutes, to ensure complete oxidation of glucose, has been recommended by Jourdonais: *J. Lab. Clin. Med.*, **23**, 847 (1937–1938); and by Horvath and Knehr: *loc. cit.*

[95] Dissolve 5 g. of picric acid in 100 ml. of methyl alcohol and add 5 ml. of 10 per cent NaOH. Place a pack of 8 to 10 heavy absorbent filter papers (Schleicher and Schull, No. 604 is good) on a level and smooth mat of newspapers. Pour the acid picrate solution over the filters until the papers are saturated and an excess of solution filters through. Allow to dry. When perfectly dry, pour over the pack an excess of a 3 per cent solution of paraffin in benzine. Allow to dry. All papers should be evenly stained. Cover the light-box window of the colorimeter lamp with stained paper. Test the efficiency by comparing water and 0.2 per cent potassium ferricyanide solution, both set at 20 mm. Adjust the colorimeter so that the two fields look alike. If the filter is inadequate, equality of the fields cannot be obtained. A strong light is necessary. A filter in the form of a glass disk to be put on top of the ocular may be obtained from the Klett Manufacturing Co., New York. See Tauber: *J. Lab. Clin. Med.*, **15**, 766 (1930).

100 mg. per cent blood glucose has a density of about 0.250, permitting accurate measurement up to about 400 mg. per cent blood glucose. For higher values, or with deeper cuvettes, use 2 ml. of filtrate plus 2 ml. of water in the analysis, and multiply the results by 2. With well-prepared reagents, the blank should have a density of less than one-tenth that of the standard given; in the presence of a blood filtrate the blank density is even less, and better agreement with Beer's law is noted if the blank is neglected and the photometer set to zero density with water alone as described.[96]

Interpretation. Normal values by this method range from 75 to 105 mg. per cent blood glucose. It must be noted that, if fingertip blood is used, this type of blood is essentially arterial, and resembles venous blood only in the fasting state. Thus during absorption or in a glucose tolerance test, higher glucose values will be obtained on fingertip blood than for venous blood, although the reverse may obtain in severe diabetes. For further interpretation, see p. 570.

6. Determination of Sugar (Method of Hagedorn and Jensen):[97] ***Principle.*** The blood protein is precipitated with zinc hydroxide. The filtrate is heated with potassium ferricyanide solution and the amount of ferricyanide reduced is determined by adding an iodide solution and titrating the iodine set free with sodium thiosulfate. The principal reaction is $2H_3Fe(CN)_6 + 2HI = 2H_4Fe(CN)_6 + I_2$. The reversal of the reduction reaction is prevented by precipitation of the ferrocyanide formed as a zinc salt.

Procedure.[98] **Into a test tube (15 × 150 mm.) pipet 1 ml. of 0.1 N NaOH and 5 ml. 0.45 per cent zinc sulfate solution. A gelatinous precipitate of zinc hydroxide forms. 0.1 ml. of blood from a capillary pipet**[99] **is introduced, the**

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[96] Unpublished results of Summerson and Robinson.

[97] Hagedorn and Jensen: *Biochem. Z.*, **135**, 46 (1923) and **137**, 92 (1923). For critical studies of this method, with suggested improvements, see Folin and Malmros: *J. Biol. Chem.*, **83**, 121 (1929); Kramer and Steiner: *Biochem. J.*, **25**, 161 (1931).

[98] Reagents Required: *For Protein Precipitation: 0.1 N NaOH.—Zinc sulfate 0.45 per cent.* These solutions are best prepared every eight days by dilution of 2 N NaOH and a zinc sulfate solution containing 45 g. of the salt in 100 ml. of solution. *For Sugar Determination: Potassium ferricyanide* 1.65 g. and sodium carbonate (fused) 10.6 g. in 1000 ml. of water. Protect from light. *Iodide-sulfate-chloride solutions:* KI, 5 g., zinc sulfate, 10 g., NaCl, 50 g., water to make 200 ml. It is best to prepare the solution without iodide and add the latter to portions of the solution as required. Free iodine can be almost completely removed by filtering through thick paper. The blank will take care of smaller errors. *Acetic acid solution:* 3 ml. of acetic acid (iron-free) with water to make 100 ml. *Starch solution:* 1 g. of soluble starch dissolved in 100 ml. of saturated NaCl solution. *Sodium thiosulfate solution:* 0.7 g. sodium thiosulfate in 500 ml. of water. *0.005 N Potassium iodate:* This solution is permanent and is used to check the thiosulfate and ferricyanide solutions which keep less well. It alone need therefore be prepared very accurately. Dissolve 0.3566 g. potassium iodate (water-free) in water to make 2000 ml.

Chemicals of highest purity must be used. Sodium carbonate is best recrystallized and fused in platinum. Acetic acid should be tested for iron, as should also the zinc sulfate, sodium chloride, and potassium iodide. The iodide should also be tested for iodate. Ordinary quantities of the mixed solutions will then give no test for iodine with starch but will give a test if 0.01 ml. of ferricyanide is added. Potassium ferricyanide is prepared by washing crystals of the ordinary product with water, dissolving in water with heat, and filtering the boiling solution through paper previously carefully washed with boiling water, into an evaporating dish set in ice-cold water. The fine crystals are filtered off with suction on another washed paper and again recrystallized. Dry at 50° C. Keep away from sunlight during course of preparation.

[99] The length of the 0.1 ml. pipet from the tip to the mark should be about 10 to 12 cm. and over-all length about 20 cm. Calibrate as follows: With the pipet measure out 0.1 ml. of 0.1 N potassium iodate solution into 10 ml. of water. Add acid and KI solution in the usual way and titrate with 0.02 N thiosulfate. Some of the same iodate solution is then diluted to 0.02 N and exactly 2.0 ml. of this in 10 ml. of water titrated with the same thio-

pipet being washed out twice with the mixture and blown empty. Put in a boiling water bath for 3 minutes. Filter on a funnel of 3 to 4 cm. diameter, prepared with a small filter of washed, moistened, not tightly pressed cotton, into a test tube (30 × 90 mm.). Wash the funnel and filter with two 3-ml. portions of water. Add 2 ml. of alkaline potassium ferricyanide solution and heat in a boiling water bath for 15 minutes. Cool and add 3 ml. of the iodide-sulfate solution and 2 ml. of 3 per cent acetic acid solution. Titrate with 0.005 N sodium thiosulfate, using as an indicator 2 drops of 1 per cent solution of soluble starch in saturated sodium chloride solution.

CALCULATION. Determine the blank obtained by carrying through the whole determination but without the addition of blood.[100] Multiply A (the thiosulfate buret reading) by the factor for the thiosulfate (2.00/ml. thiosulfate required for 2 ml. 0.005 N iodate). Express this value (B) for unknown and for blank as mg. glucose by consulting the table which follows. Subtract the glucose value of the blank from the glucose value of the unknown. The difference is mg. glucose in 0.1 ml. of blood. Or calculate as follows: $2.00 - B = x$ (ml. 0.005 N ferricyanide reduced). 2.00 ml. of ferricyanide are reduced by 0.385 mg. of glucose. Therefore mg. per cent glucose $= 0.385 \times \frac{x}{2.0} \times 1000$.

Interpretation. Similar to previous method.

MILLILITERS 0.005 N $Na_2S_2O_3$ USED AND MILLIGRAMS GLUCOSE PRESENT

Ml.	*0.00*	*0.01*	*0.02*	*0.03*	*0.04*	*0.05*	*0.06*	*0.07*	*0.08*	*0.09*
0.0	0.385	0.382	0.379	0.376	0.373	0.370	0.367	0.364	0.361	0.358
0.1	0.355	0.352	0.350	0.348	0.345	0.343	0.341	0.338	0.336	0.333
0.2	0.331	0.329	0.327	0.325	0.323	0.321	0.318	0.316	0.314	0.312
0.3	0.310	0.308	0.306	0.304	0.302	0.300	0.298	0.296	0.294	0.292
0.4	0.290	0.288	0.286	0.284	0.282	0.280	0.278	0.276	0.274	0.272
0.5	0.270	0.268	0.266	0.264	0.262	0.260	0.259	0.257	0.255	0.253
0.6	0.251	0.249	0.247	0.245	0.243	0.241	0.240	0.238	0.236	0.234
0.7	0.232	0.230	0.228	0.226	0.224	0.222	0.221	0.219	0.217	0.215
0.8	0.213	0.211	0.209	0.208	0.206	0.204	0.202	0.200	0.199	0.197
0.9	0.195	0.193	0.191	0.190	0.188	0.186	0.184	0.182	0.181	0.179
1.0	0.177	0.175	0.173	0.172	0.170	0.168	0.166	0.164	0.163	0.161
1.1	0.159	0.157	0.155	0.154	0.152	0.150	0.148	0.146	0.145	0.143
1.2	0.141	0.139	0.138	0.136	0.134	0.132	0.131	0.129	0.127	0.125
1.3	0.124	0.122	0.120	0.119	0.117	0.115	0.113	0.111	0.110	0.108
1.4	0.106	0.104	0.102	0.101	0.099	0.097	0.095	0.093	0.092	0.090
1.5	0.088	0.086	0.084	0.083	0.081	0.079	0.077	0.075	0.074	0.072
1.6	0.070	0.068	0.066	0.065	0.063	0.061	0.059	0.057	0.056	0.054
1.7	0.052	0.050	0.048	0.047	0.045	0.043	0.041	0.039	0.038	0.036
1.8	0.034	0.032	0.031	0.029	0.027	0.025	0.024	0.022	0.020	0.019
1.9	0.017	0.015	0.014	0.012	0.010	0.008	0.007	0.005	0.003	0.002

7. *Manometric Methods for Reducing Sugars.*[101] See original papers.

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sulfate as before. The two titrations should agree within experimental error (about 0.5 per cent) if the pipet is accurate.

[100] Acetone and β-hydroxybutyric acid do not reduce the reagent. One mg. of uric acid gives a reduction equal to that of 0.53 mg. of glucose, and 1 mg. of creatinine a reduction equal to 0.47 mg. of glucose.

[101] Van Slyke and Hawkins: *J. Biol. Chem.*, **79**, 739 (1928); **83**, 51 (1929).

CARBOHYDRATE TOLERANCE TEST

Principle. Blood sugar is determined at hourly periods following the ingestion of 1 g. of glucose per kilogram of body weight. Urinary sugar for the 24-hour period following the ingestion of the glucose is also determined.

Procedure. **The first thing in the morning, collect a specimen of urine and one of blood to serve as controls. Then give the patient 1 g. of glucose per kilogram of body weight. The glucose may be given in 50 per cent solution. Collect three or four specimens of blood at hourly intervals and analyze for sugar. Following the taking of glucose collect a 24-hour specimen of urine and determine its sugar content. Brill[102] and others have proposed the use of a test breakfast instead of the glucose meal.**

Interpretation. In normal individuals, blood sugar rises from the normal value of about 100 mg. to about 150 mg. per 100 ml. at the end of the first hour, and returns to normal by the end of the second hourly period. In pathological conditions, the curve does not follow the normal course. Hyperthyroidism, diabetes mellitus, and nephritis show much greater values, depending on the severity of the disease, and the return to normal is delayed for three hours or more. The higher sugar concentration in the blood during the test may or may not be accompanied by glycuresis, depending upon the "threshold point" of the kidney. In diabetes the threshold point is usually, and sometimes markedly, increased above its normal range of 160 to 180 mg. per 100 ml. In hypoendocrine conditions, in which the blood sugar is low ordinarily, the curve of blood sugar during a tolerance test is quite flat. Wherever possible, it is better practice to obtain quarter-hour specimens of blood, during the first hour. This provides more information concerning the general nature of the curve and the height of the peak. John[103] believes that a diagnosis of diabetes is justified if the curve remains high after three hours, irrespective of the height to which it has risen. Petty and Stoner[104] on the other hand regard the criterion as a rise above 180 mg. They base their claim on simultaneous determinations of respiratory quotients, which show very slight rise in diabetics, but follow the sugar curves in normal individuals and in renal glycosurics. In nondiabetics the peak of the curve is usually between one-half and one hour, while in diabetics it is nearer two hours.

It has been shown by Foster[105] that carbohydrate tolerance tests conducted on finger blood, which is practically arterial, give higher and sharper curves than tests of venous blood collected simultaneously. It appears that some of the glucose of arterial blood is removed and oxidized or stored as glycogen by the tissues. The various mechanisms for glucose utilization appear to be stimulated to overactivity, since the blood sugar curve frequently falls below its initial level. For further discussion of the application of sugar tolerance tests, consult papers by Hamman and Hirschman, Hubbard and Wright, Bailey, Williams and Humphreys, Allen, Stillman and Fitz, Rabinowitch, Macleod, Foster, and du Vigneaud and Karr. (See references in footnote 105.)

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[102] Brill: *J. Lab. Clin. Med.*, **8**, 727 (1923).

[103] John: *Am. J. Med. Sci.*, **169**, 102 (1925); *J. Metabolic Research*, **1**, 497 (1922).

[104] Petty and Stoner: *Am. J. Med. Sci.*, **171**, 842 (1926).

[105] Hamman and Hirschman: *Arch. Internal Med.*, **20**, 761 (1917); Bailey: *Arch. Internal Med.*, **23**, 455 (1919); Williams and Humphreys: *Arch. Internal Med.*, **23**, 537, 546, 559 (1919); Allen, Stillman, and Fitz: *Monograph of the Rockefeller Institute for Medical Research*, No. 11, 1919; Macleod: *Physiol. Revs.*, **1**, 208 (1921); Foster: *J. Biol. Chem.*, **55**, 291, 303 (1923); Du Vigneaud and Karr: *J. Biol. Chem.*, **66**, 281 (1925); Hubbard and Wright: *Clifton Med. Bull.*, **12**, 155 (1926); Rabinowitch: *Brit. J. Exptl. Path.*, **8**, 76 (1927).

DETERMINATION OF CHOLESTEROL

Introduction. The cholesterol of blood is present in the form of both free cholesterol and cholesterol esters. In the plasma both free and esterified cholesterol is found; in the red cells only the free form appears to be present. Plasma is preferred to whole blood for analysis, since pathological variations in the amount and in the distribution between free and ester forms occur largely in the plasma fraction. Free cholesterol is best determined by precipitation and isolation as the insoluble digitonide; the precipitate may be weighed, or treated with a cholesterol color reaction, or otherwise determined. Total cholesterol is most accurately determined in a colorimetric procedure after preliminary saponification to liberate ester cholesterol from combination, since free cholesterol and ester cholesterol may give different color intensities per unit amount of cholesterol present. Many methods which do not involve saponification have, however, been proposed, as discussed below.

1. Method of Schoenheimer and Sperry:[106] ***Principle.*** An acetone-alcohol mixture is used to precipitate the proteins and extract the cholesterol and cholesterol esters from the sample of whole blood or (preferably) plasma or serum. The cholesterol is precipitated with digitonin, either before saponification (free cholesterol) or after saponification (total cholesterol), and the separated digitonide is purified and subjected to the Liebermann-Burchard color reaction. The color is then compared with that produced by a standard cholesterol solution.

Procedure.[107] **Place approximately 10 ml. of the acetone-alcohol mixture in a 25-ml. glass-stoppered volumetric flask and add 1 ml. of serum or plasma down the side of the flask below the graduation. After withdrawing the pipet,**

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[106] Schoenheimer and Sperry: *J. Biol. Chem.*, **106**, 745 (1934); Sperry, *Am. J. Clin. Path.*, **8**, Tech. Suppl. 2, 91 (1938); Sperry (personal communication). See also Fitz: *J. Biol. Chem.*, **109**, 526 (1935); Sobel and Mayer: *J. Biol. Chem.*, **157**, 255 (1945). The original Schoenheimer-Sperry method is a microprocedure, requiring but 0.2 ml. of sample. The procedure described here is a slightly modified version which uses a larger sample, requires less technical skill, and has been found satisfactory for routine use in several laboratories.

[107] Reagents Required: *Acetone-Alcohol Mixture.* Mix 1 volume of absolute ethyl alcohol with 1 volume of redistilled acetone.

Digitonin Solution. Dissolve 400 mg. of digitonin (Hoffman-LaRoche, Nutley, N. J., or S. B. Penick and Co., 50 Church St., New York, N. Y.) in 100 ml. of distilled water. Filter or centrifuge just before use if not clear.

10 Per Cent Acetic Acid Solution. Dilute 1 volume of glacial acetic acid to 10 volumes with water, and mix.

Acetone-Ether Mixture. To 1 volume of redistilled acetone add 2 volumes of peroxide-free ether and mix. To prepare peroxide-free ether, wash ordinary ether with sodium sulfite, followed by water, and distill from calcium chloride.

Potassium Hydroxide Solution. Dissolve 10 g. of reagent-grade potassium hydroxide in 20 ml. of water. Store in a bottle equipped with a medicine dropper carrying a rubber bulb.

Phenolphthalein Solution. Dissolve 1 g. of phenolphthalein in 95 per cent alcohol and dilute to 100 ml.

Acetic Acid. Only the highest purity anhydrous product ("glacial") may be used.

Acetic Anhydride-Sulfuric Acid Reagent. Just before needed, place 20 ml. of acetic anhy-

swirl the flask to produce a finely divided precipitate, then immerse the flask in boiling water, with swirling to prevent bumping, until the solvent boils. Remove, cool to room temperature, and make up to volume with the alcohol-acetone mixture. Stopper, mix thoroughly, and pour onto a dry filter, collecting the clear filtrate in a dry test tube. Cover the funnel with a watch glass during filtration, to minimize evaporation of solvent.

Precipitation of Free Cholesterol. **Transfer 6 ml. of filtrate to a 15-ml. centrifuge tube, add 3 ml. of digitonin solution, and 1 drop of 10 per cent acetic acid solution. Place a stirring rod in the tube and stir thoroughly. Place the tube, together with the stirring rod, in a pint or quart size preserving jar, cover the jar tightly, and allow to stand at room temperature overnight. In the morning, transfer the tube and rod to a test tube rack and stir gently to free particles of precipitate which may have adhered to the walls of the tube. Remove the rod carefully without touching the upper part of the tube and place it aside carefully so that no adherent precipitate is rubbed off; a wire rack with numbered positions is suggested, so that the rod may be returned to the proper tube later. Centrifuge the tube for about 15 minutes at about 2800 r.p.m. Decant the supernatant and drain in an inverted position for a few minutes, removing the last drop by touching the lip of the tube to a clean towel; or remove the supernatant by gentle suction through a fine capillary pipet, without stirring up the precipitate or touching the sides of the tube. A few particles may float at or near the top of the solution after centrifugation; they are discarded with the supernatant.**

Replace the stirring rod in the tube and wash down the walls of the tube and the rod with 1.5 to 2 ml. of acetone-ether mixture added from a dropping pipet with attached rubber bulb. Stir the precipitate up thoroughly, replace the rod on the rack, and centrifuge the tube for 5 minutes. Remove the supernatant as above, and repeat the washing twice more, using ether instead of acetone-ether. Replace the rod in the tube and set aside until ready for color development.

Saponification and Precipitation of Total Cholesterol. **Add 3 drops of potassium hydroxide solution to a 15-ml. graduated centrifuge tube, and add 3 ml. of the acetone-alcohol filtrate. Insert a stirring rod and stir vigorously until no droplets of alkali can be seen in the solution. Leave the rod in the tube, and place tube and rod in a preserving jar containing a layer of sand about 3 cm. deep which has been previously heated in a water bath until the temperature of the sand is about 45° C. The sand acts as a heat reservoir. Close the jar tightly and place in an incubator (37° to 40° C.) for 30 minutes. Remove the tube to a rack, allow it to cool to room temperature, raise the rod, and add alcohol-acetone mixture to the 6-ml. mark. Add 1 drop of phenolphthalein solution, followed by 10 per cent acetic acid, drop by drop**

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dride (Eastman) in a glass-stoppered cylinder and chill in ice water. When cold, add 1 ml. of concentrated sulfuric acid, a little at a time, with mixing and cooling during the addition. Stopper the cylinder, shake the contents vigorously for a few moments and return to the ice bath. Keep cold during use, and do not use any reagent more than 1 hour old. More or less of the reagent may be prepared as needed, using the same proportions.

Stock Cholesterol Standard. Dissolve 100 mg. of pure dry cholesterol in about 50 ml. of glacial acetic acid by warming on an electric hot plate and stirring. Transfer with rinsings to a 100-ml. glass stoppered volumetric flask, dilute to 100 ml. with acetic acid, and mix. This solution contains 1 mg. of cholesterol per ml. and is quite stable in the cold. It must be warmed to room temperature before using. *Dilute standard.* Transfer 5 ml. of stock standard, containing 5 mg. of cholesterol, to a 50-ml. glass-stoppered volumetric flask, dilute to the mark with acetic acid, and mix well. This solution contains 0.2 mg. of cholesterol in 2 ml.

with stirring, until the red color disappears. Add 1 drop in excess, followed by 3 ml. of digitonin solution. Stir thoroughly, place the tube and rod in a preserving jar, cover tightly, and allow to stand at room temperature for at least 3 hours, and preferably overnight. Separate and wash the precipitate exactly as described above for the precipitation of free cholesterol, except that only one ether washing instead of two is necessary.

Development and Reading of Color. Place a layer of sand about 3 cm. deep in a shallow pan and heat to 110° to 115° C. in an oven. Place the tubes containing the precipitated and washed cholesterol digitonide in the pan and return the pan to the oven.[108] After 30 minutes, remove the pan and, while the tubes are still in the hot sand, from a buret add 2 ml. of acetic acid in such a way that the acid washes down the wall of the tube and the rod. Stir well with the rod, allow to remain in the sand for about 2 minutes (not longer), remove and cool to room temperature, with the rod still in the tube. Undissolved material at this stage is neglected.

When the tubes are ready for color development, place them in a water bath at 25° C. from which light is excluded[109] and allow to come to temperature equilibrium. Note the time, and add 4 ml. of the cold acetic anhydride-sulfuric acid reagent, mix well with the rod, and return to the bath. Allow to stand 27 minutes, then transfer to colorimeter or photometer containers and read within the next 10 minutes. If possible, it is better to have the time of standing before reading constant at about 30–31 minutes.

A standard suitable for both colorimetric and photometric measurement is prepared as follows: Transfer 2 ml. of a standard solution of cholesterol in acetic acid, containing 0.2 mg. of cholesterol, to a suitable tube containing a stirring rod. Place in the water bath at 25° C. and allow to come to temperature equilibrium. When ready, add 4 ml. of the acetic anhydride-sulfuric acid reagent, mix, return to the bath, and allow to stand 27 minutes. Use within the next 10 minutes.[110] For photometric measurement, a blank is prepared similarly, except that 2 ml. of acetic acid alone is used instead of the standard cholesterol solution.

For colorimetric measurement, the colorimeter must be equipped with microcups and the eyepiece of the colorimeter must be provided with a red filter (Wratten No. 71A, supplied by Eastman Kodak Co. or by the manufacturer of the colorimeter). Arrange the time of color development so that the standard is ready first, and the unknowns reach the 27-minute stage at short intervals thereafter, depending upon the rapidity with which the color comparisons can be made. Adjust the standard against itself at 20 mm., and as each unknown becomes ready, rinse cup and plunger with a small portion and read against the standard. Do not use the standard after 37 minutes have elapsed from the start of color development; if more unknowns are present than can be read in this time, have a second standard suitably timed and ready for use.[111]

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[108] This heating is to ensure complete removal of water from the hygroscopic digitonide precipitate.

[109] A large pan of water containing metal racks and placed in an enclosed box fitted with a door, or in a closet, is satisfactory. Insert a thermometer in the water and keep at 25° C. by adding hot or cold water as required.

[110] Because the standard appears to fade rapidly while it is in the colorimeter cup, Fitz (*loc. cit.*) suggests the preparation of a larger volume of standard, fresh portions of which are used at intervals during the 27 to 37 minute period of color development.

[111] Because of the instability of the standard, it has been suggested that when a number of analyses are being run colorimetrically, an artificial standard be used which is standardized each day against a cholesterol standard. Shapiro, Lerner, and Posen (*Proc. Soc. Exptl. Biol. Med.*, **32**, 1300 (1935)) proposed a permanent artificial standard made from Carter's

For photometric measurement, transfer the colored solutions to suitable containers and determine the density in the photometer, at 625 mμ. Adjust the photometer to zero density against water, and determine the densities of the blank, the standard, and the unknowns at 30 to 31 minutes after adding the acetic anhydride–sulfuric acid reagent. Subtract the density of the blank from those of the standard and unknowns, to obtain the true densities.

CALCULATION. *For colorimetric measurement:* Since the amounts of filtrate taken for the free and total cholesterol determinations represent 0.24 and 0.12 ml. of original sample respectively, the calculations are as follows:

Free Cholesterol.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.2 \times \frac{100}{0.24} = \text{mg. free cholesterol per 100 ml.}$$

Total Cholesterol.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.2 \times \frac{100}{0.12} = \text{mg. total cholesterol per 100 ml.}$$

For photometric measurement:

Free Cholesterol.

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.2 \times \frac{100}{0.24} = \text{mg. free cholesterol per 100 ml.}$$

Total Cholesterol.

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.2 \times \frac{100}{0.12} = \text{mg. total cholesterol per 100 ml.}$$

At 625 mμ, and in a 1-cm. cuvette, the density of the standard is approximately 0.100[112] (Fig. 153). Under the conditions described, from 40 to 200 mg. per cent free cholesterol and 80 to 400 mg. per cent total cholesterol may be accurately determined.

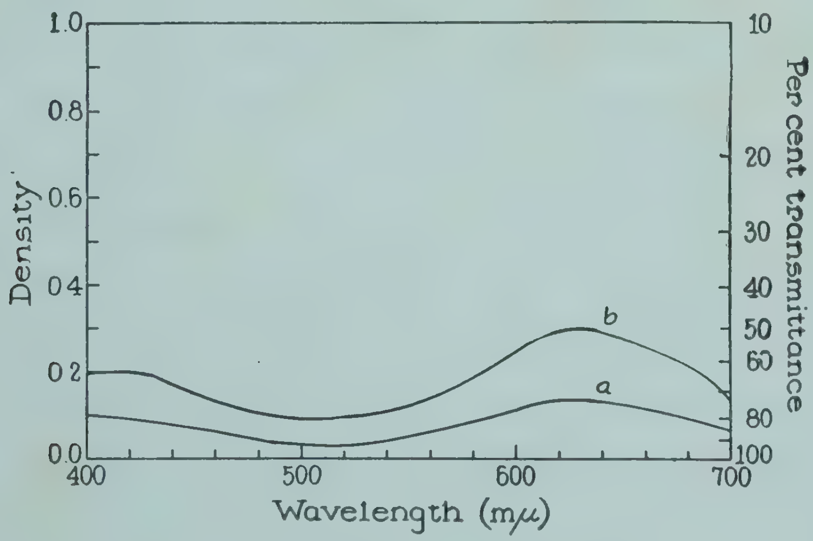

FIG. 153. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED BY SCHOENHEIMER-SPERRY METHOD FOR CHOLESTEROL.

For standards containing (*a*) 0.2 mg., and (*b*) 0.4 mg. cholesterol. Solution depth, 1 cm.

Midnight Black Ink. Sperry and Brand (*J. Biol. Chem.*, **150**, 351 (1943)) describe in detail the use of dilute naphthol green B solution as an artificial standard.

[112] Color developed for 30 to 33 minutes at 21° C.

For higher levels, repeat the analysis using less sample in the alcohol-acetone precipitation; or use less filtrate in the digitonin precipitation, with other reagents in proportion except for the final solution in acetic acid and color development; and correct the calculations accordingly.

Because of the sensitivity of the cholesterol color to bleaching by light, photometric measurements should be made as rapidly as possible, to avoid prolonged exposure to the light beam in the photometer.

Interpretation. Normal blood cholesterol appears to be maintained at a constitutional level which is characteristic for each individual, and from which large deviations do not ordinarily occur for that particular individual. Considerable variation, however, is found among different individuals, the normal range being from about 110 to 390 mg. per 100 ml. of serum or plasma. Of this, about one-third is present as free cholesterol (on the basis of digitonin precipitation, which is generally accepted as standard) and the remainder is esterified. Although many of the data in the literature are in terms of total cholesterol, the distribution of cholesterol between the free and the ester forms is attracting more attention, particularly in the diagnosis of liver disease. According to Sperry, routine determination of *whole blood* cholesterol should be abandoned, since the cells contain only free cholesterol and in quite constant amount, no changes in pathological conditions having been noted. Serum cholesterol is increased, and the determination has clinical value, in nephrosis, lipemia, diabetes mellitus, hypothyroidism, and biliary obstruction due to calculi or other causes. Increases are also found in pregnancy and after a high lipide diet. Decreased serum cholesterol is found in hyperthyroidism, pernicious anemia, and certain types of liver disease. In the latter condition, the proportion of ester cholesterol to total cholesterol may be sufficiently lowered to be of diagnostic significance. A low-cholesterol diet leads to lowered serum cholesterol; since there is ample evidence that cholesterol is synthesized within the body, changes in blood level due to diet presumably represent merely the net result of the various factors concerned.

2. Reinhold and Shiels' Modification of the Myers-Wardell Method:[113] ***Principle.*** The serum or plasma is dried on anhydrous sodium sulfate and extracted with chloroform. The total cholesterol of the extract is determined colorimetrically by the Liebermann-Burchard reaction with acetic anhydride and sulfuric acid. In the original method of Myers and Wardell, plaster of Paris was used instead of sodium sulfate. Leiboff[114] describes a procedure in which the sample is dried on a small piece of absorbent paper which is then extracted in a specially designed flask. In the Bloor, Pelkan, and Allen[115] procedure, no special apparatus is necessary; proteins are precipitated with alcohol-ether mixture, an aliquot of the filtrate is dried in a beaker and extracted with successive small portions of hot chloroform. In all of these procedures, low results will be obtained if the extraction is incomplete.

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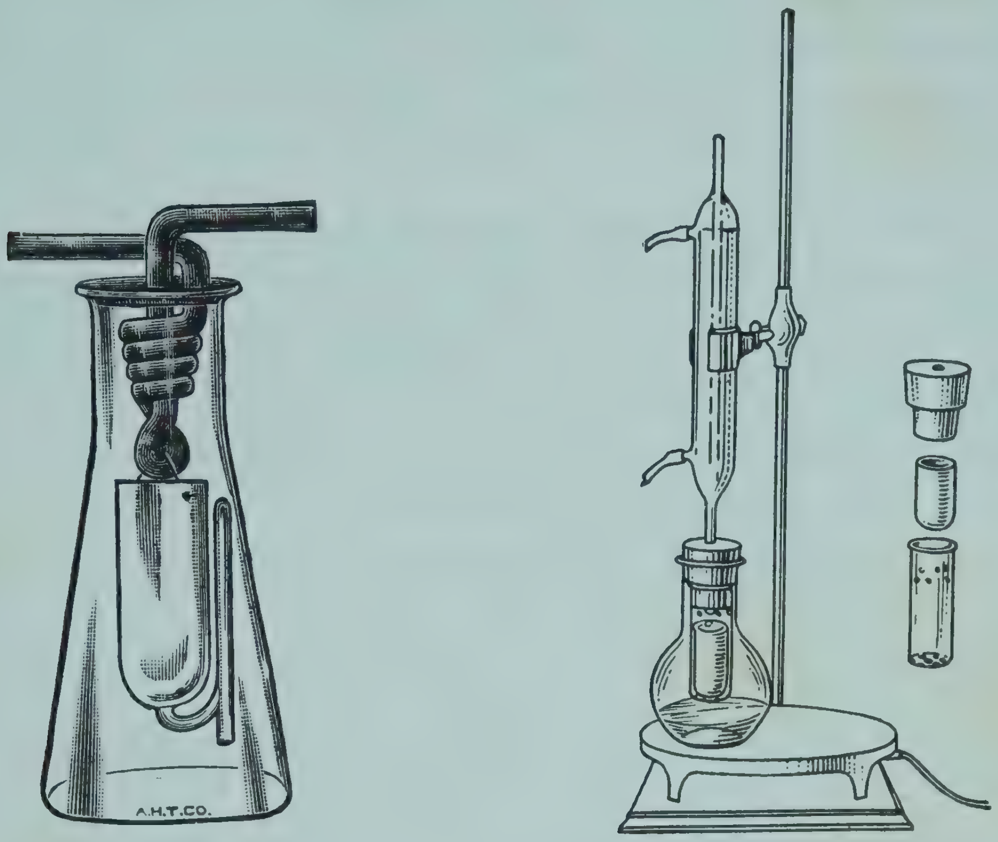
[113] Myers and Wardell: *J. Biol. Chem.*, **36**, 147 (1918); Reinhold and Shiels: *Am. J. Clin. Path.*, **6**, 22 (1936).

[114] Leiboff: *J. Biol. Chem.*, **61**, 177 (1924); *J. Lab. Clin. Med.*, **10**, 857 (1925); **11**, 777 (1926); **15**, 776 (1930).

[115] Bloor, Pelkan, and Allen: *J. Biol. Chem.*, **52**, 191 (1922). See also Kelsey: *J. Biol. Chem.*, **127**, 15 (1939).

Procedure.[116] **Transfer 1 ml. of plasma or serum to a small mortar containing about 8 g. of anhydrous sodium sulfate. Mix uniformly, dry in an oven at 100° C. for 10 minutes, cool in a desiccator, pulverize, and transfer completely to a paper extraction shell which is then inserted into a Soxhlet extraction tube (see Fig. 154) suspended from the condensing coil of the extraction apparatus. (If the extraction apparatus illustrated in Fig. 154 is not available, Myers' arrangement shown in Fig. 155 may be used, in which the extraction tube is a perforated glass tube (2.5 × 7 cm.) and is connected to a reflux condenser as illustrated.) Place 20 to 25 ml. of redistilled chloroform in the extraction flask, place on an electric hot plate, and extract for 90 minutes, during which time cold water flows through the condenser or coil. Allow the extract to cool and transfer with rinsings to a 25-ml. volumetric flask. Make up to volume with chloroform, mix well, and filter through a dry filter if necessary.**



FIGS. 154 AND 155. EXTRACTION APPARATUS FOR CHOLESTEROL DETERMINATION.

Fig. 155 after Myers: *Practical Chemical Analysis of Blood,* 2d ed., C. V. Mosby Co., St. Louis.

Transfer 10 ml. of the chloroform extract to a dry test tube and add 2 ml. of the freshly prepared acetic anhydride–sulfuric acid reagent. Treat 10 ml. of a standard solution of cholesterol in chloroform, containing 0.8 mg. of

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[116] Reagents Required: *Anhydrous sodium sulfate,* reagent grade.

Chloroform. Commercial chloroform should be washed with water, dried over anhydrous potassium carbonate, and distilled. Dry the distillate over phosphorus pentoxide and again distil. Keep protected from light. Present-day reagent-grade chloroform (such as Merck's) may be satisfactory without the necessity of further treatment.

Acetic Anhydride–Sulfuric Acid Reagent. Just before use, add 1 volume of concentrated sulfuric acid slowly with shaking to 10 volumes of acetic anhydride. Prepare only enough reagent for a particular series of analyses, and discard the unused portion.

Cholesterol Standard. Stock Standard. Dissolve 160 mg. of pure dry cholesterol in chloroform and transfer with washings of chloroform to a 100-ml. flask. Make up to volume with chloroform and mix. This solution contains 8 mg. of cholesterol in 5 ml. Keep cold and away from light. To prepare the dilute working standard, transfer 5 ml. of stock standard to a 100-ml. volumetric flask, dilute to the mark with chloroform, and mix. This solution contains 0.8 mg. of cholesterol in 10 ml. It is stable for some days if kept cold and away from light.

cholesterol, in the same way, and for photometric measurement prepare a blank by treating similarly a 10-ml. portion of chloroform alone. After mixing, keep in a dark place at 25° C. for exactly 30 minutes,[109] then transfer to colorimeter or photometer containers and read.

For colorimetric measurement, compare the unknown against the standard in the usual way. The colorimeter may be equipped with a light filter over the eyepiece (Wratten No. 71A or its equivalent, supplied by Eastman Kodak Co. or by the manufacturer of the colorimeter) although this is not essential. Since the standard color fades rapidly, if more than two or three unknowns are to be read, a number of standards must be prepared, suitably timed with respect to the unknowns, or the standard may be used to standardize an artificial standard (see previous method) against which the properly timed unknowns are read.

For photometric measurement, determine the densities of the blank, standard, and unknown at exactly 30 minutes after adding the acetic anhydride sulfuric acid reagent, in a photometer at 660 mμ. Set the photometer to zero density with water. Subtract the density of the blank from the other values to obtain their true densities.

CALCULATION. *For colorimetric measurement:* Since the 10 ml. of extract used correspond to 0.4 ml. of original sample, the calculation is as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.8 \times \frac{100}{0.4} = \text{mg. cholesterol per 100 ml.}$$

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.8 \times \frac{100}{0.4} = \text{mg. cholesterol in 100 ml.}$$

Under the conditions specified, and in a 1-cm. cuvette, the density of the standard is approximately 0.700. Since the standard corresponds to a serum with 200 mg. per cent cholesterol, the limit of accurate measurement under these conditions is about 300 mg. per cent. It is suggested for photometric measurement that smaller aliquots, such as 2 ml. or 5 ml., of the chloroform extract be taken, diluted to 10 ml. with chloroform, and the color then developed as described. Using the above calculation formula, results must then be multiplied by 5 or 2 respectively. In this way a much wider range of cholesterol concentration may be accurately covered.

Discussion. The final color in the above procedure is due to both free cholesterol and cholesterol esters. Since free cholesterol and ester cholesterol give different amounts of color per milligram, and the color develops at different rates, interpretation of results may be obscure if an abnormal distribution of cholesterol between free and ester forms is present. Reinhold[117] utilizes the difference in velocity of the Liebermann-Burchard reaction with cholesterol esters and free cholesterol as a basis for determination of cholesterol partition. Sperry and Brand[118] describe a procedure for total cholesterol after saponification of the ester fraction, thus eliminating errors due to variation in ester content.

The Liebermann-Burchard reaction with cholesterol gives much more color in chloroform than in the glacial acetic acid used in the previous method. To offset this advantage, the reaction in chloroform is, according to Schoenheimer and Sperry, more sensitive to the effect of such analytical variables as time, temperature, etc., so that more careful technical control is necessary. The spectrophotometric characteristics of the color in chloroform are somewhat similar to those of the color in acetic acid (see Fig. 153) except that peak light absorption in the red end of the spectrum is at 660 mμ instead of at 625 mμ.[119] The high light absorption at 420 mμ permits use of this range as well as the 660 mμ range. Although the color intensity here slowly increases rather than waxing and waning as at 625 mμ, and is a function of the extent of exposure to light during color development, neverthless Summerson and Robinson[120] found this range to be in some respects more satisfactory than at 660 mμ. Sunderman and Razek[119] have shown that measurement at 530 mμ is also satisfactory; although the optical density is low here, the agreement with Beer's law is excellent.

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[117] Reinhold: *Proc. Soc. Exptl. Biol. Med.*, **32**, 614 (1935).

[118] Sperry and Brand: *J. Biol. Chem.*, **150**, 351 (1943).

Interpretation. See under previous method.

Other Methods. Other methods for the determination of serum cholesterol usually employ isolation as the digitonide, followed either by weighing on a microbalance,[121] or oxidation with chromic acid. In the oxidation procedures, either titrimetric[122] or gasometric[123] methods may be employed. For details, see the original papers.

DETERMINATION OF FATTY ACIDS

Introduction. The fatty acids of blood are present largely as esters in the form of (*a*) neutral fat, (*b*) phospholipides, (*c*) cholesterol esters; there may be a small amount of free fatty acid (i.e., soaps) also present. The total fatty acids of whole blood are distributed approximately evenly between cells and plasma; this even distribution apparently simply reflects the fact that while phospholipides for example are much more abundant in the cells than in the plasma, the reverse is true for esterified cholesterol, which is apparently absent from the cells. Total fatty acid content is usually determined by saponification and titration of the liberated fatty acids.

Method of Stoddard and Drury:[124] ***Principle.*** The blood is extracted with alcohol-ether, the extract saponified, the fatty acids separated, filtered, washed, dissolved in alcohol, and titrated with thymol blue as indicator.

Procedure: EXTRACTION. **Five ml. of whole blood, plasma, or serum is introduced gradually into a 100-ml. volumetric flask containing about 75 ml. of a mixture of 95 per cent alcohol and redistilled ether (3:1). The flask is immersed in boiling water and rotated frequently and vigorously (to prevent superheating) until boiling begins, then cooled to room temperature, made up to volume with alcohol-ether, mixed, and filtered through fat-free filter paper.**

Saponification. **To a 100-ml. beaker containing a few grains of coarse sand (previously boiled with acid, washed, dried, and extracted with ether) add**

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[119] Sunderman and Razek: *J. Biol. Chem.*, **118**, 379 (1937).

[120] Unpublished.

[121] Mueller: *J. Biol. Chem.*, **25**, 549 (1916); Ewert: *Biochem. Z.*, **263**, 159 (1933).

[122] Okey: *Proc. Soc. Exptl. Biol. Med.*, **26**, 518 (1929); Turner: *J. Biol. Chem.*, **92**, 495 (1931).

[123] Kirk, Page, and Van Slyke: *J. Biol. Chem.*, **106**, 203 (1934).

[124] Stoddard and Drury: *J. Biol Chem.*, **84**, 741 (1929). This method has been critically evaluated by Man and Gildea: *J. Biol. Chem.*, **99**, 43 (1933).

gradually, while evaporating on a water bath, 75 ml. of the filtrate. The temperature should be low enough to avoid perceptible boiling but may be raised after the ether has evaporated. Evaporate to a volume of about 30 ml. Add 0.1 ml. of saturated CO_2-free NaOH,[126] mix, add a few grains more of sand, cover with a watch glass, and boil gently for 20 to 30 minutes (to saponify).

Separation and Washing. Remove the watch glass, drop in a small piece of litmus paper, make acid with 30 per cent HCl; then run back to alkalinity with 10 per cent NaOH (in order to avoid an excess of alkali on evaporating to dryness). Evaporate to dryness (in order to get rid of all alcohol). Add 15 ml. of water, heat on the steam bath, and stir to dissolve the soaps. While hot, add a drop of thymol blue indicator and make acid (faint pink) with 30 per cent HCl. Set the beaker for 10 minutes in cold water, then swirl almost continuously for five minutes to produce a better separation of the fatty acids.

Filters are previously prepared as follows: Use a Gooch crucible, smallest size (top 28 mm., bottom 18 mm. in diameter). Set the crucible in a rubber holder which fits over a 500-ml. suction flask. A paper-pulp suspension is made by shaking up a piece of soft filter paper in 300 to 400 ml. of distilled water. Shake vigorously and immediately pour some into the crucible while there is a strong suction on. Repeat until a layer about 1 mm. thick is formed. Tamp the layer down carefully all over with the end of a glass rod. Allow the larger masses of filter pulp fibers in the suspension to settle out, and pour on successive amounts of the thin upper suspension of isolated shreds, keeping a strong suction on and tamping down occasionally, until the filter is dense enough to offer a definite resistance to the suction. Remove the crucible from the rubber holder and dry in an air oven at 110° C. for 15 minutes. Allow to cool before using.

Place the crucible in its holder in an ordinary funnel and filter some of the fatty acid suspension into a test tube. If the filtrate is not perfectly clear, put it through the crucible again. If the filtration does not start in a few minutes, transfer the crucible and rubber holder to the filter flask and start the suction very gently, with a test tube under the funnel. After filtration has started, continue without suction. After the fatty acid suspension is filtered and drained, wash with 4 ml. of 5 per cent NaCl solution, neutralized to methyl red. Use a pipet and run the salt solution down the walls of the beaker all around; then, tipping the beaker, use a fine bent glass rod to rinse the side of the beaker more thoroughly with the solution; then pour this rinsing into the Gooch crucible, rinsing its side with the aid of the rod. Wash until the filtrate from one washing takes not more than 0.05 ml. of 0.02 N NaOH to neutralize it to phenolphthalein. Usually this is true of the third washing. Put the crucible back on the suction flask with a nonprotein nitrogen tube (cut off to a convenient height and calibrated at 1-ml. intervals from 10 to 15 ml.) under the funnel. Wash down the walls of the beaker with 5 ml. of 95 per cent alcohol, heat to boiling, and pour into the crucible. With the glass rod quickly loosen up any fatty acid fragments on the wall of the crucible. Allow to run nearly out, then put on a moderate suction. Rinse out the beaker and crucible twice more with 3 ml. of alcohol each time, heating it to boiling. Then wash off the outside of the crucible and the funnel.

Titration. Add a few grains of sand, boil the filtrate for 1 minute, cool in a beaker of water, note the volume of alcohol, add 3 drops of 0.3 per cent thymol blue in 50 per cent alcohol, titrate with 0.02 N NaOH to a pure blue which stays practically unchanged (no yellow tinge) while shaking for 2

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[126] See Appendix.

minutes, keeping a stopper in the mouth of the tube to avoid absorption of CO_2. For a blank boil 10 ml. of alcohol and titrate.

CALCULATION. Calculate the correction necessary for the amount of alcohol present before titration. Add a correction amounting to 0.005 ml. for each ml. of NaOH used in titration (a simple correction for the volume of solution). Subtract the total correction from the titration. Multiply by the normality factor, thus getting the number of millimoles of fatty acid. Multiply by

$$\frac{100}{\text{ml. filtrate evaporated}} \times \frac{100}{\text{ml. blood used}} = \text{millimoles fatty acid per 100 ml. blood}$$

To translate into terms of weight (not a very significant figure), multiply by an average factor for the fatty acids as they usually occur in blood = 277.2. The molecular weights are so nearly alike that a considerable variation in the proportions will not affect the calculated weight by more than about 2 per cent.

Interpretation. Human whole blood has a total fatty acid content ranging from about 9 to 14 millimoles (milliequivalents) per liter. This corresponds to about 250 to 390 mg. of average fatty acids per 100 ml. Some variation is found between different individuals, and in a particular individual at various times. Factors such as diet and disease which influence the blood content of neutral fat, phospholipide, and cholesterol esters will naturally influence the total fatty acid content.

DETERMINATION OF LIPIDE PHOSPHORUS

Introduction. The lipide phosphorus of blood and tissues is found in such compounds as lecithin, cephalin, sphingomyelin, phosphatidyl serine, etc., which are obtained by the extraction of tissues with certain nonaqueous solvents. In blood plasma, lecithin is the major phospholipide according to Bloor, while in the red cells cephalin and sphingomyelin predominate; the data of Kirk[126] present a somewhat different picture. The simplest procedure for the determination of phospholipide is the analysis of lipide-containing extracts for total phosphorus, as described below; this procedure gives fairly accurate results. Bloor[127] isolates phospholipides from nonlipide phosphorus by precipitation with acetone and magnesium chloride, and determines the phospholipide titrimetrically after oxidation with chromic acid. Gasometric determination as total carbon by wet combustion is also satisfactory.[128] Kirk[126] and Artom[126] have described procedures for the separate microdetermination of certain of the various phospholipides in blood.

Method of Youngburg,[129] Modified: Principle. The extracted lipides are oxidized with sulfuric acid and hydrogen peroxide and the phosphate present determined colorimetrically. The phosphate procedure of Fiske and SubbaRow is applied here; the Youngburgs use the stannous chloride reagent of Kuttner and Cohen (see p. 630) which is applicable to smaller amounts of phosphorus.

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[126] Kirk: *J. Biol. Chem.*, **123**, 623, 637 (1938). See, however, Sinclair and Dolan: *J. Biol. Chem.*, **142**, 659 (1942); Artom: *ibid.*, **157**, 595 (1945).

[127] Bloor: *J. Biol. Chem.*, **82**, 273 (1929). For an adaptation of this method, see Ellis and Maynard: *J. Biol. Chem.*, **118**, 701 (1937). McCoy and Schultze (*J. Biol. Chem.*, **156**, 479 (1944)) describe a photometric adaptation of the chromic acid oxidation procedure, suitable for small amounts of lipides.

[128] Kirk, Page, and Van Slyke: *J. Biol. Chem.*, **106**, 203 (1934); Van Slyke and Folch: *ibid.*, **136**, 509 (1940).

[129] Youngburg and Youngburg: *J. Lab. Clin. Med.*, **16**, 158 (1930).

Procedure. Transfer 18 ml. of alcohol-ether mixture[130] to a wide-mouthed test tube (best 150 by 20 mm.) graduated at 20 ml., and drop in slowly, while shaking, 1 ml. of plasma or serum. Mix, place in a boiling water bath, and heat the contents of the tube to boiling. Remove and allow to cool to room temperature. Make up to the 20-ml. mark with alcohol-ether mixture, mix, and filter.

Transfer 8 ml. of filtrate to a 200 by 25 mm. pyrex test tube, add a silica pebble (from broken silica ware), place in a wire rack containing a wire bottom, over an electric hot plate, and evaporate to dryness.

Add 2.5 ml. of 5 N sulfuric acid to the residue in the tube and digest over the hot plate as in the method for total acid-soluble phosphorus (see p. 633), including oxidation with perhydrol (30 per cent hydrogen peroxide). The remaining procedure is the same as for total acid-soluble phosphorus of blood (see p. 633), with color development at a final volume of 25 ml.; a different standard is however used, containing only half as much inorganic phosphate, i.e., 0.5 ml. of standard phosphate solution, containing 0.04 mg. of inorganic phosphate, instead of the 1 ml. specified on p. 634. The conditions for colorimetric or photometric measurement of the color intensity are the same as for acid-soluble phosphorus.

CALCULATION. Since the 8 ml. of extract represent 0.4 ml. of original sample, calculation of results is similar to that for acid-soluble phosphorus except that the value 0.04 replaces 0.08 in the calculations, corresponding to the use of half as strong a standard. Or calculate as for acid-soluble phosphorus and divide the result by 2, to obtain mg. of lipide phosphorus per 100 ml. of original sample.

Interpretation. Plasma or serum contains about 9 to 10 mg. of lipide phosphorus per 100 ml.; whole blood slightly more, averaging about 12 mg. per cent. These values may be expressed as lecithin by multiplying by 25, since lecithin contains approximately 4 per cent phosphorus; it is known, however, that only part of the total blood phospholipides is represented by lecithin. Artom (*loc. cit.*) gives the following distribution of phospholipides for a sample of human plasma: lecithin, 55 per cent of the total phospholipides; phosphatidyl ethanolamine (cephalin), 21 per cent; sphingomyelins, 12 per cent; phosphatidyl serine, 7 per cent. Little is known concerning the pathological significance of variation in this distribution. Pathological variation in total phospholipide content is greater for red cells than for plasma. In diabetes and nephritis, the lipide phosphorus follows roughly the degree of lipemia. Increases in lecithin are also noted in pregnancy and in certain hepatic conditions. The existence of a constant ratio between cholesterol (which is antihemolytic) and lecithin (hemolytic) has not been definitely established.

DETERMINATION OF BILE PIGMENT IN SERUM

1. Icteric Index (Meulengracht): Principle. The intensity of yellow pigmentation of serum is compared with a standard potassium bichromate solution.

Procedure.[131] Separate the serum from 4 or 5 ml. of freshly drawn unhemolyzed blood. For colorimetric measurement, accurately dilute 1 ml. of serum

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[130] *Alcohol-Ether Mixture:* Three volumes of 95 per cent redistilled alcohol and 1 volume of redistilled ether. Mix.

[131] Reagents Required: *5 Per Cent Sodium Citrate Solution.* Dissolve 50 g. of U.S.P. or C.P. sodium citrate in water and dilute to 1 liter. If turbid, allow to stand several days and

with 5 per cent sodium citrate solution in a small graduated cylinder, according to its color, until an approximate match with the standard potassium bichromate solution is obtained. Transfer to a colorimeter cup and compare against the standard bichromate.

For photometric measurement, dilute 1 ml. of serum to 10 ml. with 5 per cent sodium citrate solution. Determine the density in a photometer at 420 mμ, setting the photometer to zero density with the 5 per cent sodium citrate solution alone. Determine the density of the standard potassium bichromate solution at the same wavelength, using water as a blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{Dilution} = \text{Icteric Index}$$

For example, if 1 ml. of serum is diluted to 6 ml., the dilution is 6, and if the unknown exactly matches the standard in the colorimeter, the icteric index is 6.0.

For photometric measurement: Since the standard bichromate solution has an equivalent icteric index of 10 under the conditions of the procedure, the calculation is as follows:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 10 = \text{Icteric Index}$$

In a 1-cm. cuvette at 420 mμ, an icteric index of 10 gives a density reading of approximately 0.200, at a 1:10 dilution of the serum. Under these conditions, the limit of accurate measurement corresponds to an icteric index of approximately 50. For higher values, the determination is repeated at a greater dilution of the serum, the new dilution value replacing the 10 in the above calculation formula. Since the standard is a stable colored solution, its density should be constant. Therefore, once the density of the standard has been established for a given photometer and wavelength or filter, this value may be used in the future without the necessity of repeating the reading of the standard in every series of analyses.

Interpretation. The normal icteric index ranges between 4 and 6. The zone of latent jaundice (i.e., hyperbilirubinemia without clinical signs of jaundice) is between 6 and 15. Above this value icteric symptoms may be observed. The yellow color is considered to be due chiefly to the presence of bilirubin, an icteric index of 5 corresponding roughly to 0.1 to 0.2 mg. of bilirubin per 100 ml. of serum. The presence of certain other pigments will lead to errors; hemolysis in particular is to be avoided. This may be done by using a dry syringe and needle for collecting the blood, allowing the blood to clot protected from light, and centrifuging sharply to obtain a clear serum. Blood should be drawn before breakfast to avoid chyle; lipemic sera cannot be used because of interfering turbidity. The carotinemia which follows ingestion of carrots will lead to a high apparent icteric index and false interpretation; carrots should not be eaten the day before the test. Further aspects of the significance of serum bilirubin are discussed below.

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filter. Add a little chloroform as a preservative. Saline solution (0.9 per cent sodium chloride) may also be used as a diluent in the colorimetric procedure, but citrate gives clearer solutions.

Standard Potassium Bichromate (0.01 Per Cent). Dissolve exactly 0.1 g. of reagent-grade potassium bichromate in water, transfer with rinsings to a 1-liter volumetric flask, add 2 to 3 drops of concentrated sulfuric acid, dilute with water to the mark, and mix. Keep in a brown glass bottle. The icteric index of this solution is arbitrarily defined as equal to 1.

2. *Van den Bergh Test:*[132] *Principle.* Plasma or serum is treated with Ehrlich's reagent (diazotized sulfanilic acid). Bilirubin present reacts with the reagent to form a colored compound known as *azorubin* or *azobilirubin.*[133] Qualitatively, the color may develop immediately ("direct reaction") or on standing ("indirect reaction"). Occasionally an atypical color develops almost immediately ("biphasic reaction"). Quantitatively, the color may be used as a measure of the bilirubin content of the serum or plasma (see Section 3, p. 593).

***Procedure.*[134] Obtain unhemolyzed plasma or serum as described above. Jaundiced serum must be diluted 1:5 or, if highly colored, 1:10, with water. To 1 ml. of serum (generally diluted), add 2 ml. of freshly prepared Ehrlich's diazo reagent.[135] *Direct reaction:* a pink to purple color develops immediately, reaching maximum intensity in a minute or so. *Indirect reaction:* no color change is noted during the first 2 minutes. Within 10 minutes a golden color forms, changing over a period of an hour or more through brown to pink.[136] *Biphasic reaction:* a color appears during the first 2 minutes, but it is brownish-red rather than the typical pink or purple of the direct reaction.**

Interpretation. The chemical and clinical significance of the three types of Van den Bergh reaction has been the object of considerable investigation. Van den Bergh believed that the different reactions were dependent upon the path by which the pigment entered the serum. For example, the direct reaction is usually found in the condition of obstructive jaundice, i.e., when the biliary passages are blocked, preventing the excretion of bile pigment which has already been secreted by the liver cells. The indirect reaction is usually found when the jaundice is of hemolytic or extrahepatic origin, and the biphasic reaction is thought to represent a combination of both types. Further study has not justified the belief that the various types of jaundice may be distinguished in terms of the qualitative Van den Bergh test.[137] While normal sera and sera from cases of hemolytic jaundice usually show no direct-reacting bilirubin, in most other types of jaundice it is usually possible to demonstrate the presence of both direct-reacting and indirect-reacting bilirubin. It appears probable that there are two distinct types of serum bilirubin; for instance, indirect-reacting bilirubin may be removed from serum by extraction with chloroform, while direct-reacting bilirubin is not soluble in this solvent. Griffiths[138] has reported the isolation from human bladder bile of a pigment called "cholebilirubin" which gives the direct reaction, is distinct chemically from bilirubin, and appears to have the properties of the direct-reacting bilirubin of serum. It has been proposed that the terms "cholebilirubin" and "hemobilirubin" be used for the direct-reacting and indirect-reacting bilirubin respectively; these terms carry the unfortunate implication of origin, which has not been conclusively established. Other interpretations of the Van den Bergh reaction which have been proposed include the theory that variations are due to concentration differences,[139] or that the bilirubin of plasma is bound to protein, with a portion capable of dissociating to give an immediate reaction ("direct-reacting") while the remainder ("indirect-reacting") can react only slowly or after the addition of alcohol.[140] No single theory has apparently found universal acceptance.[141]

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[132] Van den Bergh: *Presse méd.*, **29**, 441 (1921); Sepulveda and Osterberg: *J. Lab. Clin. Med.*, **28**, 1359 (1943). See also Ducci and Watson: *J. Lab. Clin. Med.*, **30**, 293 (1945).

[133] Pyrroles couple with diazotized sulfanilic acid if they have free α or β positions or substituted groups that are readily displaced, such as carboxyl. (The α carbon is that adjacent to the N in the ring). It is believed that bilirubin is split into two dipyrryl compounds, neoxanthobilirubic acid and isoneoxanthobilirubic acid, by the diazo reagent. Each of these has a free α position which permits coupling to occur. The term azobilirubin is loosely used to describe the mixture of azo pigments produced.

[134] Reagents Required: *Diazo Reagent—Solution A.* To 1 g. of sulfanilic acid add 15 ml. of concentrated hydrochloric acid. Add water, stir to dissolve, and dilute to 1 liter with water. *Solution B.* Dissolve 0.5 g. of sodium nitrite in water and dilute to 100 ml. Just before the test, prepare the *diazo reagent* by mixing 25 ml. of Solution A with 0.75 ml. of Solution B. This mixed solution keeps only a short time at room temperature.

[135] Better results are sometimes obtained by adding the diazo reagent slowly down the side of the test tube containing the serum so that a layer is formed over the serum. Observe the color at the interface between serum and reagent.

[136] The indirect test is frequently carried out by adding three to four volumes of alcohol to the mixture of serum and reagent after 10 minutes. The indirect color appears immediately after mixing.

[137] See Greene, Plotz, and Localio: *Arch. Int. Med.*, **61**, 658 (1938); Sepulveda and Osterberg: *ibid.*, **72**, 372 (1943); also pp. 402–404 in Bodansky and Bodansky, "Biochemistry of Disease," 2nd ed, New York, The Macmillan Co., 1952.

3. Quantitative Determination of Serum Bilirubin (***Method of Malloy and Evelyn***)***:***[142] ***Principle.*** The principle is the same as that used in the qualitative Van den Bergh reactions described above. The "azobilirubin" formed is determined by photometric measurement.

Procedure.[143] **Dilute 1 ml. of unhemolyzed plasma or serum to 10 ml. with water, and mix. *Blank:* Place 5 ml. of absolute methyl alcohol in a test tube. Add 1 ml. of the blank hydrochloric acid solution, mix by tapping, and add 4 ml. of the 1:10 diluted serum or plasma. Mix gently by inversion. *Unknown:* Place 5 ml. of absolute methyl alcohol in a second test tube. Add 1 ml. of freshly prepared diazo reagent, mix by tapping, and add 4 ml. of the 1:10 diluted serum or plasma. Mix gently by inversion. Care must be taken to handle both blank and unknown tubes in the same manner, so that any turbidity which may form will be the same in both tubes. Allow to stand 30 minutes for color development, then transfer to suitable containers and read in the photometer at 540 mμ. Set the photometer to zero density with the blank. This compensates for any extraneous color or turbidity present in the unknown.**

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[138] Griffiths: *Biochem. J.*, **26**, 1155 (1932). See also Najjar: *Pediatrics*, **10**, 1 (1952); Cole and Lathe: *J. Clin. Path.*, **6**, 99 (1953).

[139] Snider and Reinhold: *Am. J. Med. Sci.*, **180**, 248 (1930).

[140] Coolidge: *J. Biol. Chem.*, **132**, 119 (1940); Martin: *J. Am. Chem. Soc.*, **71**, 1230 (1949).

[141] For review see Gray: *The Bile Pigments*, New York, John Wiley & Sons, 1953.

[142] Malloy and Evelyn: *J. Biol. Chem.*, **119**, 481 (1937).

[143] Reagents Required: *Absolute Methyl Alcohol.*

Diazo Reagent: Solution A. To 1 g. of sulfanilic acid add 15 ml. of concentrated hydrochloric acid, dissolve and dilute with water to 1 liter. Stable indefinitely. *Solution B.* Dissolve 0.5 g. of sodium nitrite in water and dilute to 100 ml. Keep away from light, and discard when it turns yellow. *Diazo Reagent.* Mix 10 ml. of Solution A with 0.3 ml. of Solution B. Must be prepared fresh before using.

Blank Hydrochloric Acid Solution. Dilute 15 ml. of concentrated hydrochloric acid to 1 liter with water. Stable indefinitely.

Standard Bilirubin Solution: Stock Standard. Place 10 mg. of bilirubin (obtainable from Eastman Kodak Co., Rochester, N.Y., or Hoffmann-La Roche, Nutley, N.J.) in a 100-ml. volumetric flask, add chloroform to dissolve, and dilute to 100 ml. with chloroform. Keep in a dark bottle in the cold. *Dilute standard.* Transfer 10 ml. of stock standard to a 100-ml. volumetric flask and dilute to the mark with methyl alcohol. Mix. This standard contains 0.01 mg. of bilirubin per ml. Prepare just before using.

CALCULATION.

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. bilirubin in standard} \times \frac{10}{4} \times 100$$
$$= \text{mg. bilirubin per 100 ml. serum or plasma}$$

The density of the standard is established as follows: Transfer 4 ml. of dilute standard alcoholic solution of bilirubin, containing 0.04 mg. of bilirubin, to a test tube, and add 5 ml. of methyl alcohol, followed by 1 ml. of diazo reagent. Mix by inversion and allow to stand 30 minutes for color development. At the same time, prepare a blank tube containing 9 ml. of methyl alcohol and 1 ml. of diazo reagent. Mix. After 30 minutes, set the photometer to zero density at 540 mμ with the blank tube and determine the density of the standard.

Under the conditions specified, and in a 1-cm. cuvette, the standard has a density of approximately 0.300 (see Fig. 156). Since this standard corresponds in an analysis to a serum bilirubin content of 10 mg. per cent, up to about 30 mg. per cent of bilirubin may be accurately determined. If the value is above this, dilute the contents of both blank and unknown tubes with an equal volume of 50 per cent methyl alcohol, read immediately, and multiply the results by 2.

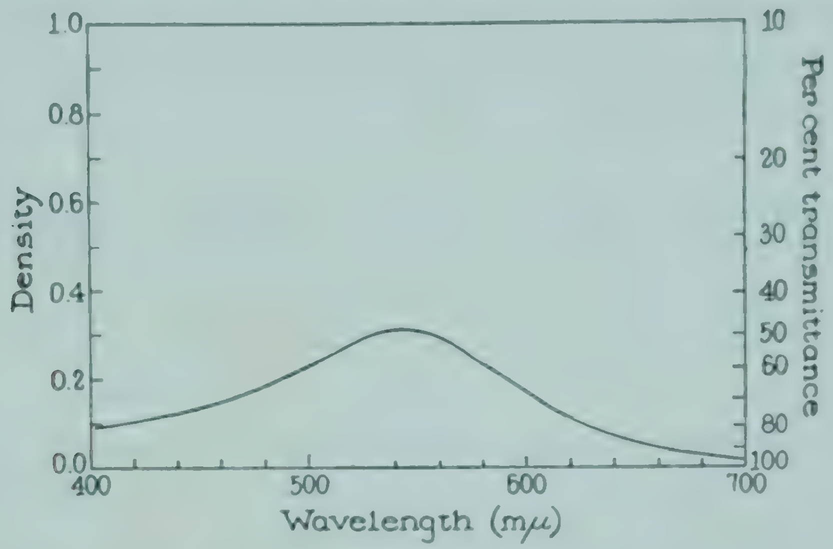

FIG. 156. ABSORPTION SPECTRUM OF AZOBILIRUBIN, AS OBTAINED IN MALLOY-EVELYN BILIRUBIN METHOD.

The color developed in this procedure is quite stable and reproducible. Since bilirubin is expensive and the standards do not keep very well, it is feasible to determine the standard density once carefully for a given photometer and wavelength setting or filter, and to use this value in future analyses, without the necessity of repeating the reading of the standard each time.

If a photometer is not available, the unknown may be compared in a colorimeter against the standard in the usual manner. A green filter over the eyepiece of the colorimeter will aid in color matching. An artificial standard containing cobalt sulfate has been suggested for visual colorimetry,[144] to replace the bilirubin standard. A colorimetric method has been described by Gibson and Goodrich.[145]

Interpretation. Normal plasma or serum contains 0.1 to 0.25 mg. of bilirubin per 100 ml. Bilirubin content is sometimes expressed in "units," 1 unit corresponding to 0.5 mg. per cent; thus normal plasma contains 0.2 to 0.5 unit. The zone of latent jaundice is represented by the range of 0.25 to 1.0 mg. per cent. Hyperbilirubinemia may occur in diseases of the liver or biliary tract, and also in extrahepatic conditions of a hemolytic nature, such as those accompanying infectious diseases, pernicious anemia, hemolytic anemia, hemorrhage, etc. Low values for bilirubin may be found in secondary anemia. The determination may therefore be of importance in the differential diagnosis of anemias. The renal threshold for bilirubin is 3.5 to 4.0 units (1.7 to 2.0 mg. per cent) in the plasma.

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[144] White: *Brit. J. Exptl. Path.*, **13**, 76 (1932).

[145] Gibson and Goodrich: *Proc. Soc. Exptl. Biol. Med.*, **31**, 413 (1934).

THYMOL TURBIDITY AND FLOCCULATION TEST

Principle. Maclagan[146] discovered that thymol, in barbital buffer at low ionic strength, added to serum produced marked turbidity in the presence of parenchymatous liver disease. In addition, flocculation often appeared on longer standing.

The thymol-barbital reagent is saturated with thymol. Temperature and other factors influencing solubility must be considered in preparing the solution. The procedure described[147] permits better control of thymol concentration and pH and yields a more uniform reagent of improved stability.

Procedure.[148] **Measure 6 ml. of thymol-barbital reagent into a photometer cuvette. Place the tubes in a water bath maintained at 25° C. ± 1°. Add 0.1**

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[146] Maclagan: *Brit. J. Exp. Path.*, **25**, 234 (1944).

[147] Reinhold and associates. Personal communication.

[148] Reagents Required: *Thymol-Barbital Reagent.* Transfer 6.0 g. of thymol crystals (colorless) to a 2000-ml. pyrex Erlenmeyer flask. Heat 1000 ml. of distilled water to boiling in another flask and boil it for about five minutes to remove carbon dioxide. Allow the water to cool to about 95°, and pour about 300 ml. into the flask on the thymol, which will melt and partially dissolve. Add 3.09 g. barbital and 1.69 g. sodium barbital and the remainder of the hot water to the flask containing the thymol. Without delay, stopper the flask and mix by rotating vigorously for about five minutes. Allow the solution to cool gradually to room temperature. Transfer the reagent to a volumetric flask of 1000 ml. capacity and dilute to the mark with distilled water. Return the solution to the original flask. Add about 1 g. of thymol crystals. Shake vigorously until the solution becomes clear. Allow the flask to stand at 25 ± 1° overnight preferably in a constant temperature bath. Mix again and filter through Whàtman No. 1 paper, maintaining the temperature of the solution at or near 25° C. Test the pH which should be 7.55 ± 0.03. The pH may be adjusted by adding 0.1 N NaOH if the reagent is too acid, or by shaking in the presence of a little CO_2 if too alkaline. Expired air may be used as a convenient source of CO_2.

The optimal temperature at which to keep the reagent appears to be 15°, however at a room temperature of 25° C., the reagent remains unchanged for at least two weeks. At temperatures lower than 15° it is more stable but crystals may separate thus causing lower values for thymol turbidity. Remove sufficient solution for one day's use and place it in a bath at 25° C. at least 30 minutes before using. Exposure to carbon dioxide should be kept to a minimum. The reagent should be renewed when it becomes opalescent.

Standards. Turbidity can be measured either visually or photometrically. The latter is recommended. For photometric measurement, colloidal glass suspensions may be used as standards. These may be obtained commercially or prepared according to the following procedure: Fill a glass-stoppered pyrex reagent bottle of 1-liter capacity to about one-fourth its capacity with fragments of clean pyrex glass. Cover the glass fragments with distilled water and agitate vigorously on a mechanical shaker until a milky suspension is produced; 8 to 20 hours is ordinarily sufficient. Decant the suspended glass into a 1000 ml. cylinder and dilute to the mark with distilled water. Mix and allow to stand 14 days. Decant the upper 500 ml. for use as a stock standard.

Standardization. For this operation a Beckman Model DU spectrophotometer is satisfactory; other instruments of comparable quality should serve as well. Make a trial reading of stock standard in the spectrophotometer at a wavelength of 660 mμ in a 10-mm. cuvette, using water as a blank. Add sufficient water to an aliquot of the stock standard to make the absorbancy of the mixed diluted suspension approximately 0.100. Now measure the exact absorbancy of the diluted suspension. An absorbancy of 0.100 has been found to be equivalent to 3.3 Maclagan units and 6.5 Shank-Hoagland units in the Evelyn photoelectric colorimeter with a 660 mμ filter, 6 ml. aperture, and standard reflector. Thus, in the calculation of results, where $S = 0.100$, $C = 3.3$ (Maclagan) or 6.5 (Shank-Hoagland). If S is slightly above or below 0.100, the appropriate value for C may be calculated by direct proportion. For colorimeters other than the Evelyn instrument, it is advisable to standard-

ml. of serum from a pipet that will deliver accurately between marks. (Do not blow out the pipet.) Stopper the cuvette and mix well. In 30 minutes measure the absorbancy (optical density, see p. 513) at 660 mμ, using thymol-barbital solution for the zero setting. If the thymol turbidity exceeds 20 units (see below), repeat using 0.05 ml. of serum. Multiply the result by 2 when this is done.

CALCULATION. Thymol turbidity units = $U \times \frac{C}{S}$ where U is the absorbancy of the unknown, S of the glass standard, and C is the thymol turbidity equivalent of the standard.

For visual measurement, the egg albumin-gelatin-formazine standards of Kingsbury *et al.*, developed for the determination of protein in urine (see p. 929) were originally used by Maclagan. Comparison against these standards should be made with light coming from behind the observer. Calculate results by dividing the protein equivalent of the standard most closely matching the unknown by 60 to give Maclagan units and by 30 to give Shank-Hoagland units.

Thymol Flocculation. **Decant the turbid solution into a conical centrifuge tube of 15 ml. capacity. Stopper and replace in the water bath at 25° C. until the following day. Examine the solution for the presence of a flocculum and note also the extent to which the supernatant fluid has cleared. Flocculation is graded on a scale from 4+ (water clear) to 1+ (flocculation and clearing are minimal).**

Interpretation: a. THYMOL TURBIDITY. Results may be expressed either as Maclagan units or as Shank-Hoagland units. One Maclagan unit equals two Shank-Hoagland units. The Commission on Liver Disease of the Armed Forces Epidemiological Board has recommended that Shank-Hoagland units be adopted in preference to Maclagan units. Ninety-five per cent of a group of healthy subjects tested by Reinhold and associates were found to have thymol turbidities not exceeding 5.5 Shank-Hoagland units, and 99 per cent were below 6.6 units, using the procedure described here.

The thymol turbidity test is among the tests most frequently positive in virus hepatitis, and is particularly useful during the recovery period for evaluation of progress and for detection of carriers of viral hepatitis. Although patients with cirrhosis may show elevated thymol turbidity, it may fail to become positive in cirrhosis either of the Laennec or biliary type.

The thymol turbidity value is consistently within the limits of normal in biliary obstruction of recent origin; older lesions may occasionally result in abnormal values. Since the reagent also reacts with serum lipides, blood should be obtained from the patient in the postabsorptive state, and confirmatory evidence sought when serum lipides are elevated. Thymol turbidity may also be increased in any disease characterized by an elevated serum γ-globulin content, quite independently of liver involvement; such conditions include multiple myeloma, lymphogranuloma, sarcoidosis, parasitic infections, and others. While the liver may also be involved, proof can only be obtained by recourse to other liver-function tests not directly dependent upon changes in serum protein content.

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ize the glass standard against a serum of known thymol turbidity. The glass standard deteriorates slowly but may be used for many months if protected from contamination.

b. THYMOL FLOCCULATION. Clinical experience suggests that the occurrence of flocculation depends upon some additional or different factors than does the production of turbidity.[149] In general, the significance of a positive flocculation test is the same as for abnormal turbidity. The flocculation test is less frequently positive than is the turbidity test; however, false positive tests are uncommon and the occurrence of thymol flocculation thus may be accepted with a higher degree of confidence as evidence of liver damage. The serum of healthy individuals shows no flocculation. Flocculation graded 1+ or more, therefore, is abnormal.

CEPHALIN-CHOLESTEROL FLOCCULATION TEST

Principle. Suitably diluted serum of patients suffering from liver disease forms a flocculent precipitate when treated with a suspension of cephalin and cholesterol in water.[150] The mechanism of the reaction differs somewhat from that of the thymol turbidity test; the change in serum responsible for the test appears to be primarily in the albumin fraction.[151]

Procedure.[152] **It is advantageous to use conical centrifuge tubes of 15 ml. capacity. Measure into such a tube 4 ml. of 0.85 per cent sodium chloride solution. Add 0.2 ml. of serum and mix well by tapping the tube vigorously 5 times. Add 1 ml. of cephalin-cholesterol reagent and again mix by tapping the tube vigorously at least 15 times. Place the tube in a water bath at approximately 25° C., in the dark and away from chemical fumes, until the following day. Examine the tube for presence of flocculation after 24 hours and again after 48 hours (optional).**

Grade the reaction as 1+ to 4+ as described under "Thymol Flocculation" (p. 596) taking into account the amount of precipitate and the transparency of the supernatant fluid. If desired, the precipitate can be removed by centrifugation, the supernatant fluid decanted, and its absorbancy measured in a

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[149] Neefe: *Gastroenterology*, **7**, 1 (1946).

[150] Hanger: *J. Clin. Invest.*, **18**, 261 (1939).

[151] Moore, Pierson, Hanger, and Moore: *J. Clin. Invest.*, **27**, 737 (1948).

[152] Reagents Required: *Cephalin-Cholesterol Stock Solution.* The purchase of the reagent ("antigen") is recommended (Difco and Wilson brands are satisfactory). It is supplied in vials containing 100 mg. of cephalin, prepared from brain and aged, and 300 mg. of cholesterol. The contents of a vial are dissolved according to directions in U.S.P. ethyl ether, after addition of 1 drop of distilled water. The stock solution so obtained may be kept for several months if refrigerated and tightly stoppered.

Cephalin-Cholesterol Reagent. Measure 32 ml. of water into an Erlenmeyer flask of 50 ml. capacity with a mark scratched on it to indicate 27 ml. Place the flask on a water bath on an electric heater and heat to 65° or 70° C. While the water in the flask is kept at this temperature, add drop by drop 1 ml. of the stock cephalin-cholesterol solution, rotating the flask at the same time. Place the flask directly on the hot plate at low heat and boil gently to remove the ether until the volume is reduced to 27 ml. When cool, add 3 ml. of merthiolate solution, aqueous 1:1000, as a preservative. This reagent retains its activity unchanged for at least a week if stored in a refrigerator.

Precautions. Preparations of the cephalin-cholesterol reagent as purchased or prepared have been found to vary widely in sensitivity. Each lot of reagent must be assayed by testing serum of healthy individuals and of a sampling of patients showing positive reactions. Acceptable reagents do not produce more than an occasional 1+ flocculation when the sera of healthy subjects are tested, and on the other hand yield a high proportion of positive flocculations in patients suffering from liver disease. Once a suitable reagent has been obtained, new lots can be tested by comparing them with previous ones. For best results, tests should be done in duplicate, using two different preparations. Difficulties of standardizing and of controlling this test make it practical only when a considerable flow of samples through the laboratory may be expected.

photometer at 660 mμ, using a tube containing sodium chloride solution and reagent, but without serum, as a blank.[153]

Interpretation. With properly prepared reagents, reactions of 2 + or greater offer evidence of disturbed liver function. Hanger believes that a positive test is an indication of active parenchymal disease; in support of this may be cited the high proportion of patients suffering from acute viral hepatitis who exhibit positive cephalin-cholesterol flocculation. Positive tests occur within a few days of onset of clinical illness, frequently before jaundice or significant elevation of bilirubin appears, thus offering valuable support for a diagnosis of viral hepatitis, particularly in hepatitis without jaundice. However, failure of the cephalin-cholesterol test to become abnormal does not exclude the diagnosis. In one study, 20 per cent of a large group of patients ill of viral hepatitis gave negative tests.[154] Abnormal cephalin-cholesterol flocculation readings may or may not persist into the stage of recovery. In this respect the test differs from the thymol test, which tends to remain positive for a considerable time.

A high proportion of positive tests is found in cirrhosis. Positive tests are prevalent in a variety of diseases such as malaria, pneumonia, infectious mononucleosis, and others causing damage to the liver. Biliary obstruction of short duration is characterized by negative cephalin-cholesterol flocculations, and the test may be used along with other observations to aid in determining whether jaundice is due to surgically remediable causes. Inflammatory disease of the bile ducts usually is accompanied by a positive test.

BROMSULFALEIN TEST[155]

Principle. A measured amount of bromsulfalein is injected intravenously. The liver rapidly removes the dye from its combination with plasma proteins and excretes it into the bile. If liver function is impaired, excretion is delayed and a larger proportion of the dye remains in the circulation. The dye concentration in serum is measured after addition of alkali to convert any dye present into its intensely colored sodium salt. Corrections are made for interference by turbidity and pigmentation of the serum.

Procedure:[156] PREPARATION OF PATIENTS, INJECTION OF DYE, AND COLLECTION OF BLOOD. **The test is best done in the morning. The evening meal of the previous**

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[153] Kibrick, Rodgers, and Skupp: *Am. J. Clin. Path.*, **22**, 698 (1952).

[154] Neefe, Gambesia, Gardner, and Knowlton: *Am. J. Med.*, **5**, 600 (1950).

[155] Reinhold and Hutchinson: Unpublished.

[156] Solutions: *Sodium hydroxide solution.* Approximately 0.5 N and 0.05 N. The 0.05 N solution is prepared from 0.5 N by diluting tenfold with water.

Phenol Tetrabromosulfonphthalein Sulfonate Sodium (Bromsulfalein) Standard Solution. Transfer 50 mg. to a 500 ml. volumetric flask. Add 250 ml. of the 0.05 N sodium hydroxide solution and dilute to volume with water. (If crystalline bromsulfalein is not available, substitute 1 ml. of 5 per cent solution from an ampoule.) The standard solution so prepared contains 10 mg. in 100 ml. and is equivalent to 100 per cent retention of dye when 5 mg. per kg. is injected. (It is assumed that plasma volume represents 5 per cent of the body weight.) This standard is further diluted by measuring 10, 25, and 50 ml. aliquots and diluting them to 100 ml. with 0.05 N sodium hydroxide. The resulting standard solutions ar equivalent to 1.0, 2.5, and 5.0 mg. per 100 ml. and represent 10, 25, and 50 per cent of th initial dye concentration. The standards remain unchanged over long periods. For actua measurement 0.5 ml. of each working standard is diluted to 6 ml. with 0.05 N sodiu hydroxide.

Apparatus: A photoelectric photometer is preferred, to permit accurate measurement

day should not include foods rich in fat. A breakfast of unbuttered toast and coffee, tea (without cream), or fruit juice is permissible, after which the patient should not eat until the test has been completed.

The quantity of bromsulfalein required is calculated according to the patient's ideal weight. The accompanying table shows the volume of 5 per cent solution needed. This volume is introduced into a sterile syringe which is made ready for the injection.

VOLUME OF BROMSULFALEIN SOLUTION (5 PER CENT) REQUIRED FOR PATIENTS ACCORDING TO STATURE, BUILD, AND SEX, BASED ON TABLES OF IDEAL WEIGHTS FOR INDIVIDUALS 25 YEARS AND OVER

MEN

	Small Frame		*Medium*		*Large*	
Height	*Weight*	*Ml.*	*Weight*	*Ml.*	*Weight*	*Ml.*
5 ft. 2 in.	120 lb.	5.4	128 lb.	5.8	136 lb.	6.2
3	123	5.6	131	5.9	138	6.3
4	127	5.8	135	6.1	142	6.4
5	131	5.9	139	6.3	146	6.6
6	134	6.1	142	6.4	150	6.8
7	138	6.3	146	6.6	155	7.0
8	141	6.4	150	6.8	159	7.2
9	145	6.6	154	7.0	163	7.4
10	149	6.8	158	7.2	167	7.6
11	153	6.9	162	7.3	172	7.8
6 ft. 0	158	7.2	167	7.6	176	8.0
1	163	7.4	171	7.8	181	8.2
2	169	7.7	177	8.0	186	8.4
3	174	7.9	182	8.3	191	8.7

WOMEN

	Small Frame		*Medium*		*Large*	
Height	*Weight*	*Ml.*	*Weight*	*Ml.*	*Weight*	*Ml.*
4 ft. 11 in.	107 lb.	4.8	114 lb.	5.2	122 lb.	5.5
5 ft. 0	108	4.9	116	5.3	124	5.6
1	110	5.0	118	5.4	126	5.7
2	113	5.1	121	5.5	129	5.8
3	116	5.3	124	5.6	132	6.0
4	119	5.4	128	5.8	136	6.2
5	122	5.5	131	5.9	139	6.3
6	126	5.7	134	6.1	143	6.5
7	129	5.8	138	6.3	147	6.7
8	132	6.0	141	6.4	151	6.8
9	136	6.2	145	6.6	155	7.0
10	139	6.3	149	6.8	158	7.2
11	142	6.4	152	6.9	161	7.3

A vein is entered and about 5 ml. of blood withdrawn. This blood is used as a control specimen if necessary. The syringe containing dye is then attached to the needle and the dye injected slowly over a 3-minute period. Forty-five minutes later about 5 ml. of blood is collected from a vein of the *opposite*

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borderline retention and to permit correction for interference from hemoglobin. However, approximate measurements can be made by means of visual comparators. When the latter are used, additional standard solutions are prepared so as to give concentration intervals of 0.5 mg. up to 3.0 mg. and at 1.0 mg. intervals above this.

arm by means of a different syringe and needle. The exact time of completing the injection and of collecting the blood should be noted. The blood is allowed to clot, and serum is removed for analysis. Care is used to avoid hemolysis. Needle and syringe should be dry, and the blood should be transferred from syringe to tube gently and without production of foam.

Measurement of Dye Concentration. Measure 0.5 ml. of the serum of the dyed specimen into a cuvette containing 5.0 ml. of water. Add 0.5 ml. of 0.5 N sodium hydroxide solution. Mix well and measure the absorbancy (optical density) at 660, 565, and 420 mμ, using water for the zero settings at each wavelength. Maximum absorption occurs at 580 mμ and this wavelength setting is theoretically to be preferred; however, interference from hemoglobin is less marked at 565 mμ. If serum bilirubin is elevated, the control sample is diluted and absorbancy measured as described for the dyed sample. The figures so obtained are subtracted from those of the bromsulfalein-containing serum. Calculate results as described below.

Calculation: Bromsulfalein, mg. per 100 ml. $= f \times (U_{565} - 1.28\ U_{660}) - 0.15 \times (U_{420} - 1.95\ U_{660})$. The values for U_{565}, U_{660}, and U_{420} represent absorbancies of the diluted serum at the wavelengths of the subscripts.[157] The value for f is obtained as follows: determine the absorbancy, S, for each of the three standards (representing 1.0, 2.5, and 5.0 mg. per 100 ml.) at 565 mμ. Calculate f as follows:

$$f = \frac{C}{S_{565}}$$

where C is the concentration of standard and S_{565} is the absorbancy. Average the values to give the value of f used in the calculation. Once f has been determined, standards can be dispensed with except for occasional instrument checks. From the bromsulfalein content of the serum, the per cent retention is calculated as follows:

$$\text{Per cent retention} = \text{bromsulfalein, in mg. per 100 ml.} \times 10$$

A correction is applied in the method described to compensate for the presence of hemoglobin in serum, since hemolysis of some erythrocytes may be difficult to avoid and hemoglobin will cause falsely high readings even at 565 mμ. The effect of lactescence in serum is eliminated by making the absorbancy measurement at 660 mμ, where neither bromsulfalein nor hemoglobin absorb light. A correction may be used when the test is applied to jaundiced patients.[158]

Rosenthal and White used 2 mg. of dye per kg. of body weight. The 5 mg. per kg. dosage, however, has been adopted widely and provides a more sensitive and more precise test. The existence of an enterohepatic circulation of bromsulfalein[159] does not appear to influence the results sufficiently to necessitate a return to the original dosage.[160] The time at which the postinjection blood specimen is collected is not of great importance, provided that appropriate normal standards for that time are used; the 45-minute sampling time has proved to be satisfactory.

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[157] The correction factors given in the calculation were calculated from readings with an Evelyn photocolorimeter (see p. 526). They may be applied to readings made with other photometers provided the transmittancies at the wavelengths used do not differ greatly from those obtained with the Evelyn instrument. If desired, the factors may be determined by measuring the absorbancy A of a dilute solution of hemoglobin containing roughly 10 to 20 mg. hemoglobin per 100 ml. at 420 and 565 mμ. The correction for hemolysis is calculated by means of the ratio A_{565}/A_{420} obtained from the readings of the hemoglobin solution. This corresponds to the factor 0.15 in the calculation. The turbidity correction is based on readings of a colloidal glass suspension, similar to that described under thymol turbidity (see p. 595), made at 420, 565, and 660 mμ. Although turbid solutions have no absorption bands, their absorbancy increases progressively from the red to the blue regions of the spectrum. Therefore, the ratio A_{565}/A_{660} of the glass suspension represents the factor by which any measurements made at 660 mμ must be multiplied, and A_{420}/A_{660} the corresponding factor for the reading at 420 mμ in order to correct for turbidity. In the calculation above, these ratios are 1.28 and 1.95 respectively. The turbidity correction will vary with particle size and therefore represents an approximation. No corrections need be applied to the standards.

[158] Zieve, Hanson, and Hill: *J. Lab. Clin. Med.*, 37, 40 (1951).

Interpretation. Healthy individuals, after injection of 5 mg. bromsulfalein per kg. of body weight, retain less than 10 per cent at 30 minutes and 7.0 per cent at 45 minutes. At 60 minutes, no dye is retained.

Bromsulfalein retention is generally accepted as the most sensitive and dependable among the laboratory procedures currently used to demonstrate involvement of the liver. It is especially helpful for evaluating suspicious or positive results obtained by means of flocculation tests in the absence of hyperbilirubinemia. The bromsulfalein test outranks all others in the proportion of positive tests found in Laennec's cirrhosis. It has been among the most useful for following recovery from virus hepatitis and for detecting residual liver damage from this disease. It is probably the only procedure capable of detecting fatty liver, although it cannot be depended upon to do so consistently.

For the study of the jaundiced patient the bromsulfalein test has little to offer. Maximal retention occurs in the presence of severe liver damage, and further deterioration of the liver function can have no additional effect on dye retention. Thus it is rarely used when hyperbilirubinemia or clinical jaundice are present.

DETERMINATION OF PLASMA PROTEINS

Introduction. The standard method for the determination of protein is by the Kjeldahl type of digestion and oxidation, converting the nitrogen present to the form of ammonia, which is then determined. If the material contains nonprotein nitrogen, this is determined in a separate analysis and subtracted from the total nitrogen value to give the protein nitrogen. This is then multiplied by 6.25 to give the protein value, since the average protein contains 16 per cent nitrogen. If sufficient material is available the most accurate version of this procedure is a macro-Kjeldahl method similar to that described in Chapter 31 for the determination of the total nitrogen of the urine. For blood analysis with its inherent limitation in the amount of material available, this is replaced by the various micro-Kjeldahl methods, and the procedures become similar to the determination of nonprotein nitrogen already described (p. 545).

To obtain satisfactory results more rapidly and simply than is possible by micro-Kjeldahl methods, various direct colorimetric, turbidimetric, and specific gravity procedures have been developed. The colorimetric procedures are usually based on color reactions specific for protein or constituent amino acids and are standardized accordingly. Turbidimetric methods rely on the comparison of the turbidity produced by precipitating reagents on proteins in dilute solution with the turbidity produced similarly on a standard protein solution. The specific gravity methods are based on the fact that protein is by far the most abundant solid constituent of blood, and hence the specific gravity should be determined largely by the protein content.

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[159] Lorber and Shay: *Gastroenterology*, **20**, 262 (1952).

[160] Owen: *J. Lab. Clin. Med.*, **38**, 583 (1951).

Fractionation of the plasma proteins prior to separate analysis is based almost entirely on the classical methods using concentrated salt solutions, and the bulk of clinical literature is in terms of such fractions. Fractionation by electrophoretic mobility has not yet reached the stage of routine practicality, except possibly in a few of the larger institutions. For a further discussion of the various methods of fractionating the plasma proteins, see Chapter 22. The determination of blood hemoglobin content will be considered in a separate section.

1. Micro-Kjeldahl Method:[161] ***Principle.*** *Total proteins* are determined in serum or plasma by a micro-Kjeldahl method employing direct nesslerization, making the appropriate correction for nonprotein nitrogen. *Fibrinogen* in plasma is determined by isolation as fibrin, followed by digestion and direct nesslerization. *Albumin* is determined by analysis of the fluid remaining after precipitating the globulin fraction with 23 per cent sodium sulfate solution. *Globulin* in serum is estimated by subtracting the albumin from the total protein content; in plasma by subtracting albumin and fibrinogen from total protein.

Procedure:[162] ***Total Proteins (Albumin + Globulin + Fibrinogen).*** **Dilute exactly 1 ml. of the serum or plasma to 50 ml. in a volumetric flask with 0.9 per cent sodium chloride solution. (If serum is used, the "total protein" will**

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[161] This method is a combined adaptation of the procedures of various authors. The direct nesslerization micro-Kjeldahl procedure of Wong (*J. Biol. Chem.*, **55**, 427 (1923)), employing persulfate for oxidation, is recommended because of its superiority when protein is present. Fractionation of the proteins with sodium sulfate solution follows the procedure of Howe (*J. Biol. Chem.*, **49**, 109 (1921)); this method is significantly improved by the use of ether to aid in separating the albumin and globulin fractions, as suggested by Kingsley (*J. Biol. Chem.*, **133**, 731 (1940)). Isolation of fibrinogen as fibrin is essentially according to Cullen and Van Slyke (*J. Biol. Chem.*, **41**, 587 (1920)). These latter authors point out the desirability of using a uniform amount of anticoagulant such as oxalate in comparative studies on plasma proteins, because of the effect of oxalate on water distribution between cells and plasma. They use 5 mg. of potassium oxalate per ml. of blood. For other methods of digestion and fractionation see Campbell and Hanna: *J. Biol. Chem.*, **119**, 1, 9, 15 (1937); Pillemer and Hutchinson: *ibid.*, **158**, 299 (1945). These latter authors use cold methyl alcohol for fractionation and claim their fractions agree closely with those obtained by electrophoretic separation.

[162] Reagents Required: *0.9 Per Cent Sodium Chloride Solution.* Dissolve 9 g. of reagent-grade low-nitrogen sodium chloride in water, dilute to 1 liter, and mix. Stable indefinitely.

2.5 Per Cent Calcium Chloride Solution. Dissolve 25 g. of anhydrous reagent-grade calcium chloride in water, dilute to 1 liter, and mix. Stable indefinitely.

23 Per Cent Sodium Sulfate Solution. Dissolve 230 g. of anhydrous reagent-grade sodium sulfate in 600–700 ml. of water by warming and stirring. While still warm transfer to a 1-liter volumetric flask, dilute with water to the mark, and mix. Transfer to a clean bottle and place in the incubator or water bath at 37° C. Keep at this temperature at all times, stoppered to prevent evaporation, since some of the salt will crystallize out if kept at room temperature.

1:1 Sulfuric Acid. Pour slowly and with stirring 1 volume of concentrated sulfuric acid into 1 volume of water. Cool, and keep stoppered to prevent absorption of ammonia from the air.

Persulfate Solution. Shake about 8 g. of reagent-grade nitrogen-free potassium persulfate in a glass-stoppered bottle with about 100 ml. of water. The undissolved excess settles to the bottom and helps keep the solution saturated even though there is gradual decomposition. Keep in the refrigerator, shake briefly and allow to settle before using, and prepare fresh every few weeks.

Standard Nitrogen Solution and *Nessler Reagent.* See p. 545.

represent only albumin + globulin.) Using 1 ml. of this diluted mixture, proceed as directed below under "Digestion."

Fibrinogen. To 1 ml. of plasma in a small cylinder add 30 ml. of 0.9 per cent sodium chloride solution, followed by 1 ml. of 2.5 per cent calcium chloride solution. Mix with a slender pointed glass rod, leaving the rod in the mixture. Allow to stand for 30 minutes or until a solid clot has formed. Carefully rotate the rod in the jelly, squeezing out the water by pressing the clot against the side of the cylinder at the same time. All the fibrin should stick to the rod and appear ultimately as a thin white sheath over the rod. If any bits of clot escape this process, pour the cylinder contents onto a dry filter, and when the fluid has drained pick up the remaining clots with the tip of the rod and squeeze out the excess fluid by pressing against the side of the funnel. Transfer the rod and adhering fibrin to a piece of dry filter paper and dry as thoroughly as possible by rolling against the filter paper. Transfer the rod and dried fibrin to a centrifuge tube graduated at 10 ml. and containing 4 ml. of 1 per cent sodium hydroxide solution. Place tube and contents in a boiling water bath until the fibrin has dissolved and only a turbid suspension of calcium oxalate remains. This should require but a few minutes. Remove from the water bath, remove the rod, washing it down with a few ml. of water, and make up the contents of the tube to 10 ml. with water. Mix and centrifuge. Pipet a 5-ml. portion of the clear supernatant fluid to a digestion tube and proceed as described below under "Digestion."

Albumin. To 1 ml. of serum or plasma in a 50-ml. centrifuge tube add exactly 30 ml. of 23 per cent sodium sulfate solution. Stopper and mix by inversion. Add about 5 to 10 ml. of ether, again stopper, and shake vigorously. Centrifuge for about 10 minutes, capping the tube to prevent loss of ether. After centrifuging, the precipitated globulins should form a compact layer below the ether and above the clear albumin solution.[163] Slant the tube and insert a pipet of narrow bore, and with the mouthpiece closed by the finger, along the side of the tube past the packed globulin layer into the clear fluid below. Fill the pipet with the fluid and transfer to a dry test tube, wiping off any precipitate adhering to the outside of the pipet before discharging its contents. Use 1 ml. of this for digestion as described below. If a centrifuge is not available, the mixture may be poured onto a retentive filter (such as Whatman No. 50) and covered with a watch glass to prevent evaporation. If the first portions of filtrate are not clear, return to the filter. Use 1 ml. of the clear filtrate.

Digestion. Into a pyrex test tube graduated at 35 and 50 ml. (see method for nonprotein nitrogen, p. 545) place 1 ml. (or 5 ml. in the case of fibrinogen) of the solution to be analyzed. Add 1 ml. of 1:1 sulfuric acid and a quartz chip or a few glass beads. Digest over a microburner as described for the determination of nonprotein nitrogen until excess water has been driven off, the solution darkens, and white fumes appear. When the tube is nearly full of dense fumes, cover the mouth of the tube with a watch glass and reduce the flame or raise the tube so that the mixture boils gently. Continue boiling for 3 minutes. Remove the burner and allow to cool for 1 minute. Add to the tube contents, drop by drop, 0.5 ml. of persulfate solution. Replace the burner, tap the tube to start boiling if necessary, and con-

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[163] On cold days some of the sodium sulfate may crystallize out during the centrifuging. If this happens, warm the tube and contents in the incubator at 37° C. until the crystals have redissolved, add more ether, shake, and again centrifuge as described.

tinue boiling until clear. Cool, dilute with water, and proceed with the direct nesslerization and colorimetric or photometric measurement exactly as described on p. 546 for the determination of nonprotein nitrogen. The same standard is used, containing 0.15 mg. of nitrogen, in the presence of 1 ml. of the 1:1 sulfuric acid and 0.5 ml. of persulfate solution, to balance the amounts present in the unknown. For photometric measurement the blank tube contains water, acid, and persulfate as described for the standard.

CALCULATION. *For colorimetric measurement.*

Total Protein and *Albumin* are calculated directly, as follows:

$$\left[\left(\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.15 \times \frac{100}{V}\right) - \text{NPN}\right] \times \frac{6.25}{1000}$$

$$= \text{grams protein per 100 ml. (or per cent protein)}$$

where V represents the actual volume of serum or plasma used in the determination; NPN represents the nonprotein nitrogen content in mg. per cent, as determined in a separate analysis. For total protein, $V = 0.02$; for albumin, $V = 0.0323$ (i.e., $^1/_{31}$).

Fibrinogen is calculated as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.15 \times 2 \times 100 \times \frac{6.25}{1000}$$

$$= \text{grams fibrinogen per 100 ml. of plasma}$$

Globulin = Total Protein − (Albumin + Fibrinogen) in the case of plasma; for serum, *Globulin* = Total Protein − Albumin.

For photometric measurement. Calculations are the same except that the expression $\frac{\text{Density of Unknown}}{\text{Density of Standard}}$ replaces the expression $\frac{\text{Reading of Standard}}{\text{Reading of Unknown}}$ used in the colorimetric calculations. The limits of accurate colorimetric or photometric measurement are the same as those specified for the determination of nonprotein nitrogen on p. 547. If an analysis falls outside of these limits, the digestion is repeated, using a smaller or larger aliquot as the case may be, and the calculations are corrected accordingly.

Interpretation. Normal values for plasma proteins, in g. per 100 ml. of plasma, are as follows: albumin, 4.6–6.7; globulin, 1.2–2.3; fibrinogen, 0.3–0.6. A major function of the plasma proteins (see Chapter 22) is to aid in the normal distribution of water between the blood and tissues, albumin being approximately twice as effective as globulin in this respect, gram for gram. Edema almost invariably occurs when the total plasma proteins fall below the critical level of 5.3 g. per 100 ml. Increased plasma protein levels are noted in dehydration, due to diminished fluid intake or pathologic loss of fluid from the body (diarrhea, vomiting, surgical or traumatic shock, excessive burns, Addison's disease); or when there is an absolute increase in globulin content, as in various anaphylactic conditions, malignancy, liver cirrhosis, and certain chronic infections. Decreased plasma protein is found after loss of plasma by extravasation or renal excretion (albuminuria) or when protein synthesis is impaired owing to malnutrition, vitamin deficiencies, or diseases involving the digestive organs or liver. Fibrinogen values are increased in pneumonia and other infections accompanied by leukocytosis or suppuration, but are low in acute yellow atrophy of the liver, poisoning due to chloroform or phosphorus, and typhoid fever. High sedimentation rate of red blood cells is associated with increased fibrinogen values in plasma.

2. *Method of Kingsley:*[164] *Principle.* The diluted plasma or serum is treated with a special biuret reagent. The color developed is compared with that of a standard protein solution treated similarly. By suitable fractionation of serum before treatment with the biuret reagent, albumin may be determined separately. From the total serum protein and albumin contents, globulin is determined by difference. The method as described has been slightly modified to permit the determination of total protein, albumin, and globulin on the same sample of serum.

***Procedure.*[165] Transfer 0.5 ml. of unhemolyzed serum to a graduated centrifuge tube, or small (13 × 125 mm.) test tube graduated at 10 ml. Add 23 per cent sodium sulfate solution to the 10-ml. mark. Mix well by repeated inversion (but do not shake), and immediately pipet out a 2-ml. portion of the uniform suspension and place in a separate small test tube, which need not be graduated. This is Tube 1, which will be used for the determination of total protein. To the remainder of the suspension in the centrifuge tube add about 3 ml. of ether, stopper, and shake vigorously. Centrifuge for about 5 minutes, capping the tube to prevent loss of the ether. After centrifuging, hold the tube in a slanting position and insert the tip of a 2-ml. transfer pipet of narrow bore along the side of the tube past the white layer of packed globulin precipitate and into the clear fluid below. Remove a 2-ml. portion of the clear fluid with the pipet, wipe off any precipitate adhering to the outside of the pipet, and transfer to a second test tube (Tube 2). This will be used for the**

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[164] Kingsley: *J. Lab. Clin. Med.*, **27**, 840 (1942). See also Robinson and Hogden: *J. Biol. Chem.*, **135**, 707, 727 (1940); Mehl: *ibid.*, **157**, 173 (1945). An improved biuret reagent and procedure has been described by Weichselbaum: *Am. J. Clin. Pathol.*, **7**, 40 (1946).

[165] Reagents Required: *23 Per Cent Sodium Sulfate Solution.* Dissolve 230 g. of anhydrous reagent-grade sodium sulfate in 600 to 700 ml. of water in a beaker by heating and stirring. While still warm transfer to a 1-liter volumetric flask, dilute with water to the mark, and mix. Transfer to a clean bottle, and place in the incubator or water bath at 37° C. Keep at this temperature at all times, stoppered to prevent evaporation, since some of the salt will crystallize out if kept at room temperature.

Special Biuret Reagent. Prepare a saturated solution of sodium hydroxide which is carbonate-free (see Appendix). This saturated solution should contain about 75 g. of sodium hydroxide per 100 ml. after preparation. This may be checked by titration of a diluted portion. Measure 92 ml. of the carbonate-free saturated solution of sodium hydroxide, containing 69 g. of sodium hydroxide, into a 500-ml. graduated cylinder, and add water to the 300-ml. mark. Add 100 ml. of a 1 per cent solution of crystalline copper sulfate, and stir to mix. Transfer to a clean bottle fitted with a rubber stopper. This reagent is stable for months at room temperature. The formation of a slight sediment does not appear to impair its usefulness, particularly in photometric measurements run with a blank and standard as described in the text.

Standard Protein Solution. Obtain a pooled lot of normal human serum (10–15 ml. will be needed). Determine the total protein content of a portion by micro-Kjeldahl or macro-Kjeldahl analysis (see p. 601). Dilute 5 ml of the remainder to 100 ml. in a volumetric flask with 15 per cent sodium chloride solution, and mix. Label with the total protein content, in grams per 100 ml., of the original serum, since this value is used in the calculations. This solution is usable for about a month if kept in the refrigerator at all times. A new solution may be standardized by colorimetric comparison with the previous standard if deterioration of the latter has not occurred. If this procedure is followed, check occasionally by the Kjeldahl method to avoid the possibility of error. Since with a particular photometer the density of a given standard is quite constant if the analysis is properly carried out, this is as good an index as any of possible changes in the standard.

For colorimetric measurement, three colored standards are prepared, representing the biuret reaction on sera at a level of approximately 3, 6, and 9 g. per cent total protein respectively. These standards are stable for at least one month if stored in the refrigerator. *Standard I.* To 1 ml. of serum of known protein content add 39 ml. of water and 80 ml. of the biuret reagent. *Standard II.* To 2 ml. of the serum add 38 ml. of water and 80 ml. of biuret reagent. *Standard III.* To 3 ml. of serum add 37 ml. of water and 80 ml. of biuret reagent. The equivalent protein values for these standards, used in the calculation, are as follows: Standard I, $C/2$; Standard II, C; Standard III, $3C/2$; where C is the total protein content of the serum used, in grams per 100 ml.

albumin determination. For photometric measurement, place 2 ml. of the standard protein solution in a third tube, and 2 ml. of water alone in a fourth tube as a blank. For colorimetric measurement these latter two tubes are unnecessary since the unknown is compared against standards which have already been prepared (see below and footnote 165). To each tube add 4 ml. of the special biuret reagent, followed by 2 to 3 ml. of ether. Stopper, shake vigorously, and centrifuge for 5 minutes. By means of a pipet, transfer the solution under the ether layer to colorimeter cups or photometer cuvettes[166] and read within the next 10 to 20 minutes, preferably as soon as possible, since on prolonged standing a slight turbidity may develop which requires further treatment with ether and centrifugation. For photometric measurement, set the photometer to zero density with the blank, at 520 mμ.

If the total protein content alone is desired, the fractionation with sodium sulfate solution is omitted. Dilute 0.5 ml. of serum to 10 ml. with 0.9 per cent sodium chloride solution, mix, transfer a 2-ml. portion to a small test tube, and continue as described for Tube 1 above. Alternatively, 0.1 ml. of serum, measured in a pipet calibrated "to contain," may be pipetted into 1.9 ml. of 0.9 per cent sodium chloride solution in a test tube, the pipet being rinsed several times with the diluting fluid, and this 2-ml. portion treated with biuret reagent as described above.

CALCULATION. *For colorimetric measurement,* use a green filter (with maximal transmission at 520 mμ) over the eyepiece, set the unknown at 10 mm., and read against those two of the three standards described in footnote 165 which appear on inspection to be lower and higher respectively than the unknown. Calculate results as follows:

$$\frac{A \times \frac{R_L}{10} + B \times \frac{R_H}{10}}{2} = \text{grams protein per 100 ml.}$$

where A and B represent the equivalent protein concentrations of lower and higher standards respectively, in grams per 100 ml. of serum, and R_L and R_H are the respective standard readings. If the unknown reading is within 10 or 15 per cent of either standard, the comparison with the other standard may be omitted, in which case the calculation becomes:

$$\frac{\text{Reading of Standard}}{10} \times C \times f = \text{grams protein per 100 ml. of serum}$$

where C is the total protein concentration of the standard serum used, in grams per cent, and f is 0.5, 1.0, or 1.5 depending upon whether Standard I, II, or III is used.

Calculations for Tubes 1 and 2 are the same, the value for Tube 1 giving total protein in grams per cent, and the value for Tube 2 giving albumin in grams per cent. Globulin = Total protein − Albumin.

For photometric measurement:

Total Protein.

$$\frac{\text{Density of Tube 1}}{\text{Density of Standard}} \times C = \text{grams total protein per 100 ml. serum}$$

Albumin.

$$\frac{\text{Density of Tube 2}}{\text{Density of Standard}} \times C = \text{grams albumin per 100 ml. serum}$$

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[166] If the photometer is equipped for use with small test tubes which can be centrifuged, these may be used for color development and subsequent reading, without the necessity for transfer.

Globulin.

Total protein − albumin = grams globulin per 100 ml. serum

C is the total protein content of the (undiluted) standard serum, in grams per cent. At 520 mμ, and in a 1-cm. cuvette, a serum containing 7 g. per cent of total protein has a density of approximately 0.400, when corrected for the density of the blank (Fig. 157). Up to 11 g. per cent of total protein may be determined accurately under the conditions described. For higher values, or with deeper cuvettes, use 1-ml. portions instead of 2-ml. portions in Tubes 1 and 2, plus 1 ml. of water, and multiply the results by 2.

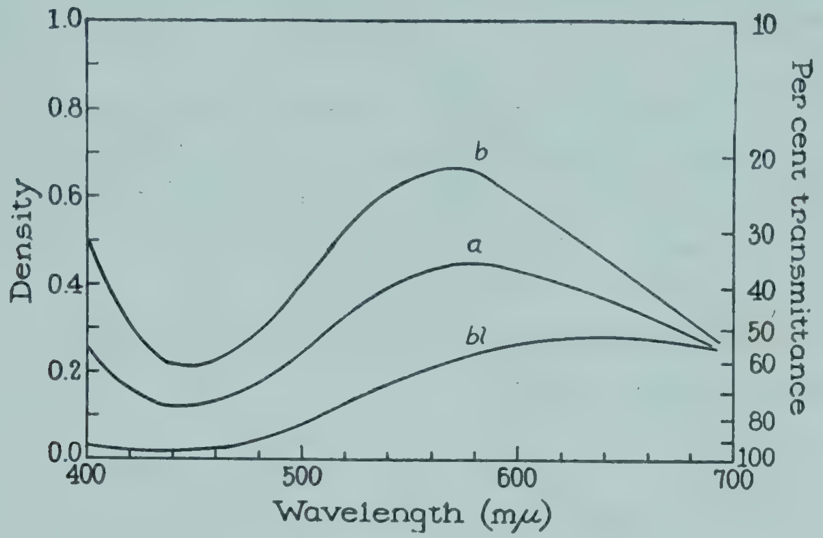

FIG. 157. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN KINGSLEY METHOD FOR PROTEIN.

For biuret reagent alone (*bl*), and for biuret reagent in presence of serum containing 3.5 g. per cent total protein (*a*), and 7 g. per cent total protein (*b*). Solution depth, 1 cm.

Interpretation. Values for most normal and pathological sera by this method show good agreement with micro-Kjeldahl determinations. Discrepancies have been noted under certain conditions, but it is not known whether this is due to alteration in nitrogen content or in biuret-reacting power. The method is known to give satisfactory clinical results. For further interpretation, see the preceding method.

3. Determination of Plasma or Serum Protein, Hemoglobin, and Cell Volume (Hematocrit) by the Copper Sulfate–Specific Gravity Method:[167]

Principle. The specific gravity of whole blood, plasma, or serum is established by allowing small drops of the material to fall into a series of copper sulfate solutions of known and varying specific gravity and noting whether the drops rise, fall, or remain suspended under the defined conditions. From the specific gravity thus established, the plasma or serum protein, hemoglobin, and hematocrit values are obtained on the basis of an experimentally established relationship between these various quantities. If the plasma or serum protein content alone is to be determined, only plasma or serum is needed. Approximate hemoglobin and hematocrit values (within about 10 per cent) may be obtained with a few drops of whole blood alone. Accurate hemoglobin and hematocrit values are obtained by determining the specific gravity of both whole blood and its plasma.

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[167] As developed by Phillips, Van Slyke, Dole, Emerson, Hamilton, and Archibald, at the Hospital of the Rockefeller Institute for Medical Research. For a "gradient tube" method, based on a similar principle but using nonaqueous solvents in a single tube, and suitable for very small amounts of serum, see Lowry and Hunter: *J. Biol. Chem.*, **159**, 465 (1945).

Procedure. Venous blood is collected with a minimal amount of stasis (tourniquet applied for not over one minute). Capillary (finger tip) blood may be used, except in shock, for the approximate hemoglobin and hematocrit determination. To obtain plasma, transfer the blood immediately after drawing to a container having either a dried film of heparin sufficient to provide approximately 0.2 mg. for each ml. of blood expected, or a dried film of the Heller and Paul oxalate mixture[168] sufficient to provide not over 1 mg. per ml. of blood expected. Mix to dissolve, and centrifuge. If both whole blood and plasma are wanted, save a portion of the well-mixed blood for the whole-blood determination and centrifuge the remainder to obtain the plasma. For serum protein determination, serum from ordinary clotted blood is used.

Prepare a series of standard copper sulfate solutions of known and varying specific gravity.[169] For the most accurate work, the series should be graded at intervals of 0.001 in specific gravity; for rougher work, intervals of 0.004 suffice. For plasma and serum, the range of specific gravity covered should be 1.015 to 1.035; for whole blood, 1.035 to 1.075.

Allow a small drop of the sample to fall by gravity from a height of about 1 cm. above the solution (see Fig. 158) into one of the standard copper sulfate solutions having approximately the specific gravity expected. The smaller the drop the better; use a medicine dropper with a drawn-out tip, or a syringe needle. If the sample is whole oxalated or heparinized blood, the cells and plasma must be thoroughly mixed by repeated inversion immediately before use, otherwise serious error will result. Observe the behavior of the drop *within the 10-second period after it has lost the momentum of its fall.* If the drop rises at all during this period, it is lighter than the test solution; if it continues to fall, it is heavier; if it remains stationary after momentum is lost, it has the same specific gravity as the solution. After the 10-second period indicated, the behavior of the drop has no significance, because of changes in specific gravity due to diffusion through the copper proteinate film around the drop. If the specific gravity of the drop is not established exactly by the first test, repeat the procedure with a fresh drop on solutions of higher or lower specific gravity as the case may be, until a solution is found in which the drop either remains stationary, which gives the specific gravity of the sample, or there are two adjacent standards in one of which the drop rises, and in the other it falls. In this case interpolation between the values of the two standards is used; by noting the relative rate of rise and fall in the two solutions, it should be possible to interpolate to one-quarter of the difference in specific gravities between them. Thus by experience one should be able to tell whether the specific gravity of the drop is halfway between the two standards, or nearer to one than to the other.

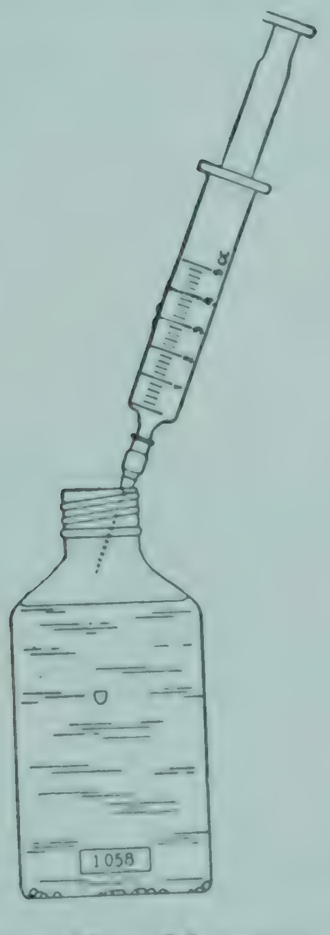

FIG. 158. METHOD OF DROPPING BLOOD INTO COPPER SULFATE.

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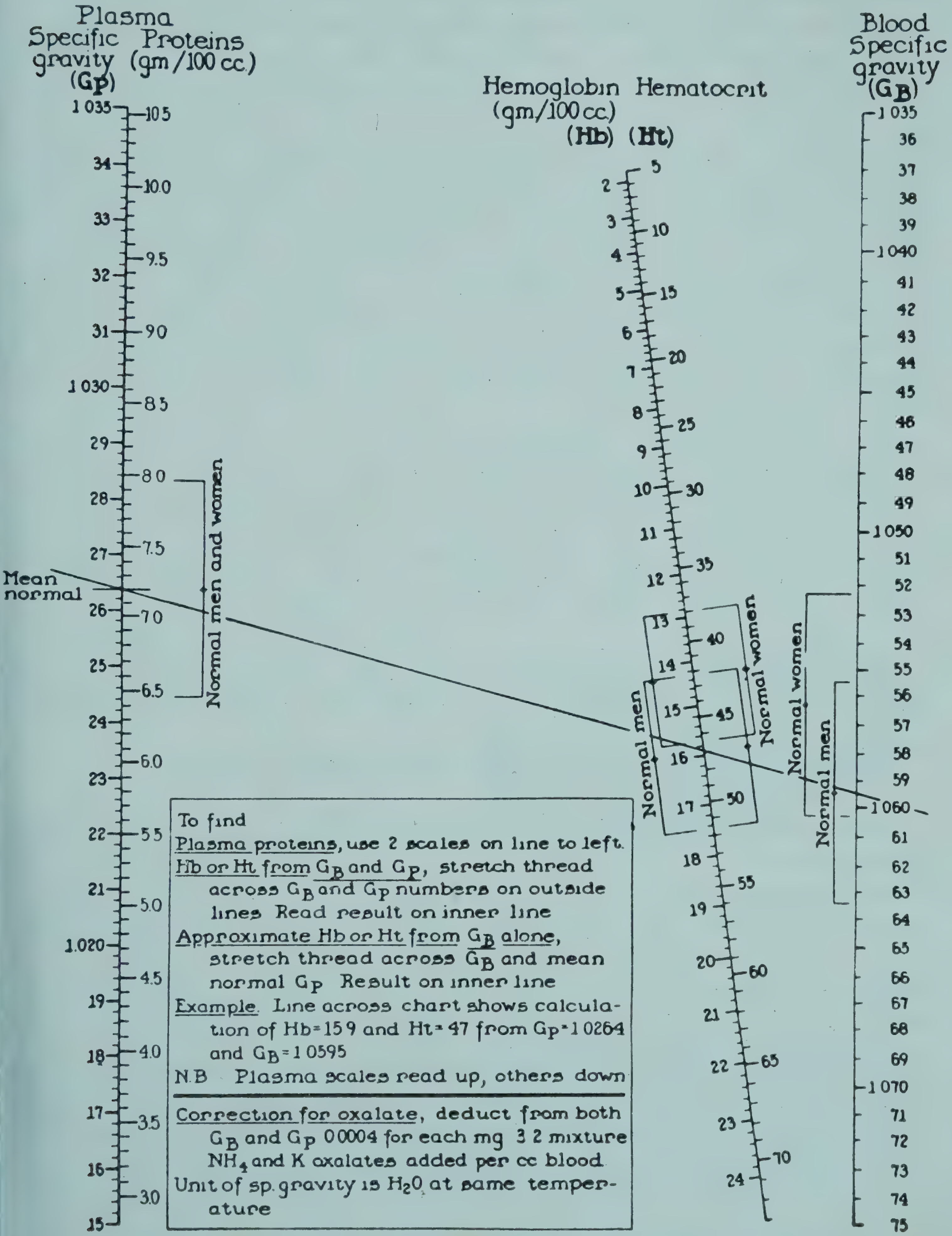
[168] Dissolve 3 g. of ammonium oxalate and 2 g. of potassium oxalate in water and dilute to 250 ml. Pipet 0.05 ml. of this solution for each ml. of blood to be received in a container (small test tube or bottle), spread in a film and dry in an incubator at 37° C. or in a vacuum desiccator. See Heller and Paul: *J. Lab. Clin. Med.*, **19**, 777 (1934).

[169] See Appendix

If oxalated whole blood and plasma are used, correct the observed values for the oxalate by subtracting 0.0004 for each mg. of oxalate mixture present per ml. of blood, applying the correction to both whole blood and plasma results. Heparinized blood or serum requires no correction. Use the corrected values in obtaining results.

CALCULATION. From the determined specific gravities, results are obtained by reference to the line chart shown in Fig. 159. For approximate hemoglobin and hematocrit



To find

Plasma proteins, use 2 scales on line to left.

Hb or Ht from G_B and G_P, stretch thread across G_B and G_P numbers on outside lines Read result on inner line

Approximate Hb or Ht from G_B alone, stretch thread across G_B and mean normal G_P Result on inner line

Example. Line across chart shows calculation of Hb=15 9 and Ht=47 from Gp=1 0264 and G_B=1 0595

N.B Plasma scales read up, others down

Correction for oxalate, deduct from both G_B and G_P 0 0004 for each mg 3 2 mixture NH_4 and K oxalates added per cc blood

Unit of sp. gravity is H_2O at same temperature

FIG. 159. LINE CHART FOR CALCULATING PLASMA PROTEINS, HEMOGLOBIN, AND HEMATOCRIT FROM SPECIFIC GRAVITIES OF PLASMA AND BLOOD.

determination using whole blood alone, the blood is assumed to have a normal plasma specific gravity of 1.0264, and a line from this point on the left-hand scale of the chart is drawn across the chart to the determined whole blood specific gravity as measured on the right-hand scale. The hemoglobin content and hematocrit value are read from the interception of this line with the middle scale. Where both plasma and whole-blood specific gravities have been determined, the line is drawn connecting the two measured values on their respective scales, and the hemoglobin and hematocrit values read from the intercept on the middle scale. For plasma protein content, in grams per 100 ml., the left-hand scale is used. If serum is used instead of plasma, the same scale is used, the results then being in terms of grams of protein per 100 ml. of serum.

The copper sulfate solutions are permanent if kept stoppered to prevent evaporation. A given standard may be used over and over again, until one-fortieth its volume of blood, plasma, or serum has been added, i.e., one small drop per ml. Thus a 100-ml. portion serves for about 100 tests. For routine work, it is good practice to keep a record of the number of drops added to a given standard, and to discard it when the indicated limit has been reached. The solutions are self-cleaning, precipitated material ordinarily settling to the bottom. If any material should stick in the surface film, either during or after an analysis, it should be dislodged by tapping or removed with a wooden applicator stick before further use. The hemoglobin of whole blood will impart a greenish color to used standards; this does not impair their effectiveness. Temperature control is necessary only in the preparation of the standards, as described in the Appendix; once the standards are prepared, further temperature control is ordinarily unnecessary.

Interpretation. See previous methods.

Other Methods. The micro-Kjeldahl method may be made more accurate by distillation followed by nesslerization or titration; gasometric determination of the ammonia may also be used. See discussion of nonprotein nitrogen determination on p. 548. The colorimetric method of Greenberg[170] is described in the eleventh edition of this book. The determination of the specific gravity is the basis of the "falling drop" method of Barbour and Hamilton,[171] which has found considerable clinical application. For a turbidimetric method, see Looney and Walsh.[172]

DETERMINATION OF HEMOGLOBIN

Introduction. In spite of the great clinical importance of hemoglobin determinations, as a rule they are the most poorly conducted of all blood chemical analyses. The majority of so-called office instruments in the hands of the average worker are known to give errors as high as 20 per cent. The very fact that a method is adapted for small amounts of blood should presuppose accurate calibration and use of pipets, careful dilution and color match, and frequent checking of both instrument and technician on blood of known hemoglobin content.

The standard method for the determination of hemoglobin, and the one upon which almost all others are ultimately based, is the determination by gasometric methods of the oxygen-binding power (*oxygen capacity*) of the blood. This method is described in detail in Chapter 24. An advantage of this method is that when properly carried out the results represent the *functional* hemoglobin of the blood, or that portion which is capable of carrying oxygen,[173] and do not include such nonfunctional pigment as methemoglobin, carbon monoxide hemoglobin, etc., as is the case with most of the other common methods. Results by the oxygen capacity method may be expressed directly in terms of oxygen, as *volumes per cent*, i.e., ml. of bound oxygen per 100 ml. of blood, or they may be converted to grams of hemoglobin by making use of the empirically established fact that 1 g. of hemoglobin is capable of combining with 1.36 ml. of oxygen under optimal conditions.[174] Thus an oxygen capacity of 20 volumes per cent corresponds to a hemoglobin content of 20/1.36, or 14.7 g. per 100 ml.

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[170] Greenberg: *J. Biol. Chem.*, **82**, 545 (1929).

[171] Barbour and Hamilton: *J. Biol. Chem.*, **69**, 625 (1926).

[172] Looney and Walsh: *J. Biol. Chem.*, **130**, 635 (1939).

Another method for the determination of hemoglobin which is also suitable for standardization purposes, particularly in laboratories where gasometric equipment is not available, is to determine the total iron content of the blood. The iron content of hemoglobin has been accurately established at 0.340 per cent,[174] and the hemoglobin iron ordinarily represents 98 per cent or more of the total blood iron. Thus if the iron content is determined, the hemoglobin content may be accurately established. A simple and reliable colorimetric procedure for this purpose, developed by Wong, is described elsewhere in this section.

Direct colorimetric procedures for hemoglobin determination range from the most simple to the most complex, with a corresponding range in accuracy. They utilize either the color of the blood itself (diluted or undiluted as the case may be) or the color produced by treating blood with various reagents. The color is compared with, or measured in terms of, a standard color obtained from or representing a blood of known hemoglobin content. These procedures are best used in conjunction with the ordinary laboratory colorimeter or photometer as described below. For clinical purposes, a number of special "hemoglobinometers" of various types have been designed. The Dare hemoglobinometer, one of the simplest of the clinical instruments, is based on comparison of a film of undiluted blood of uniform depth with a red glass plate of graduated ntensity which has been suitably standardized. In the Spencer "Hb-Meter" (Fig. 160), the absorption of light by the hemoglobin in a layer of hemolyzed blood of fixed depth is compared with light absorption of a standardized glass wedge. The widely used Sahli type of clinical hemoglobinometer (Fig. 161), is based on treatment of the blood with dilute acid to produce the brown "acid hematin" color, followed by dilution until the color exactly matches a standard brown glass plate. From the

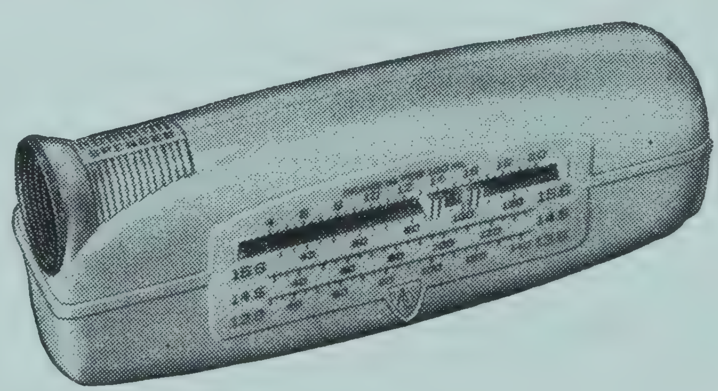

FIG. 160. AO SPENCER HB-METER.
Courtesy, American Optical Company, Buffalo, N. Y.

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[173] The desirability of this distinction may be limited under certain conditions, as for xample in methemoglobinemia. See also Rimington: *Ann. Rev. Biochem.*, **12**, 430 (1943).

[174] Bernhart and Skeggs: *J. Biol. Chem.*, **147**, 19 (1943).

dilution required, the hemoglobin content is readily obtained. This instrument is frequently very carelessly used; with care, however, it is capable of giving results accurate to within 5 per cent or so. The development of the photoelectric cell has produced hemoglobinometers of which the Fisher "Electro-hemometer" is an example (Fig. 162). This is essentially a one-purpose desk-model photoelectric photometer; the blood sample, appropriately diluted in the glass tube, is placed in the instrument and the hemoglobin content read directly from the scale, which is calibrated by the manufacturers.

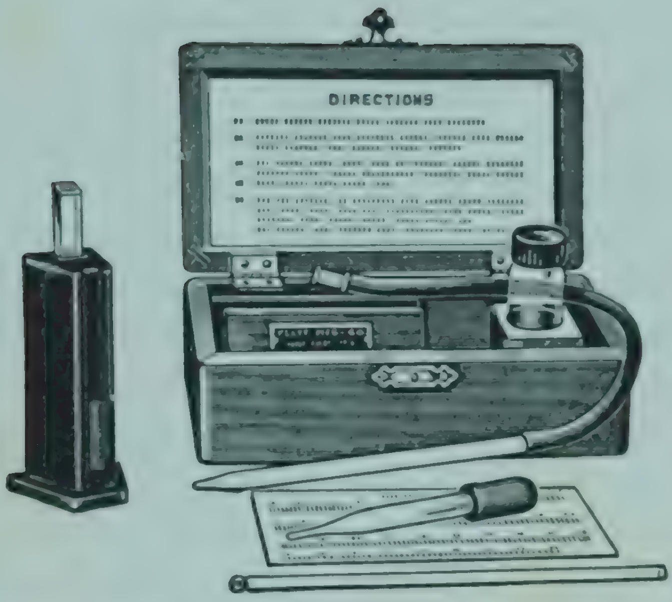

Fig. 161. Sahli Hemoglobinometer.
Courtesy, Klett Manufacturing Co.

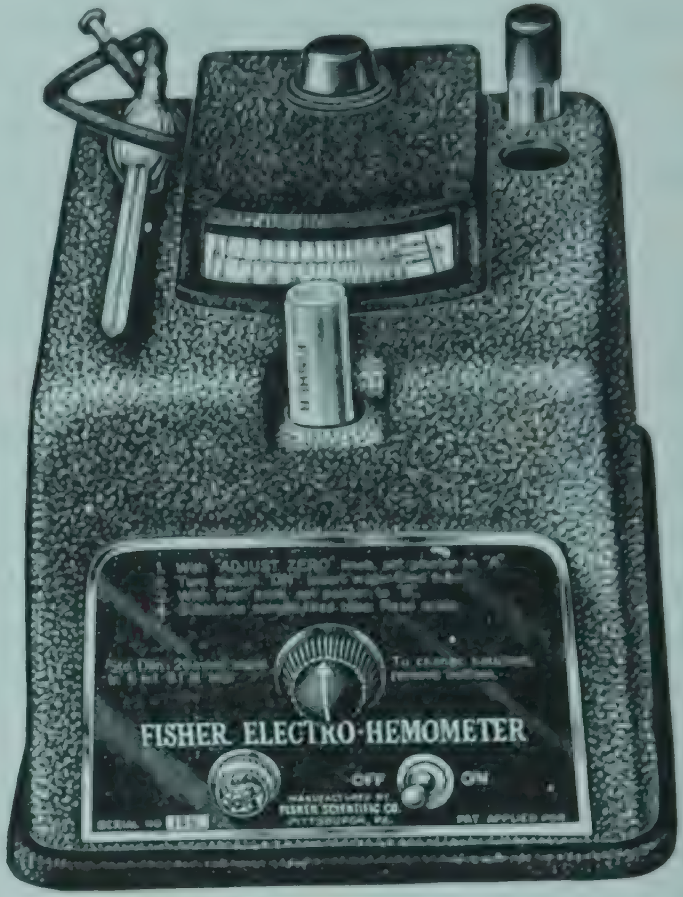

Fig. 162. Fisher Electro-hemometer.
Courtesy, Fisher Scientific Co.

For all hemoglobinometers supplied with a ready-made calibration, regardless of type or source, the desirability of checking the calibration at intervals with blood of known hemoglobin content cannot be overemphasized. Manufacturers do not use these instruments, they merely make them; and the conditions of calibration and use may vary from time to time and differ from laboratory to laboratory. Only by careful checking under the conditions of actual use is it possible to eliminate errors from this source.

It has been the custom among clinicians to express hemoglobin values in terms of per cent of some arbitrarily established normal. Unfortunately, there is no general agreement as to what the "normal" value should be, for the very good reason that the hemoglobin content of the blood of a normal individual depends upon such factors as age, sex, occupation, climate, altitude, and other environmental circumstances, etc., and is obviously also influenced by the red cell count of the blood and factors which cause normal variation in this respect. The confusion attendant to the use of "per cent of normal" is illustrated by the fact that a blood hemoglobin content of say 14.5 g. per 100 ml., which is an exact value, becomes "105 per cent of normal" on the Haldane scale, where 13.8 g. per cent is considered normal, and "84 per cent of normal" on the original Sahli scale, which considered 17.3 g. per cent as normal. The logical way to avoid this confusion, and one which is finding increasing clinical favor, is to express blood hemoglobin content in terms of grams of hemoglobin per 100 ml. of blood, or volumes per cent oxygen capacity, whichever is preferred, without reference to any arbitrary "normal," interpreting the result if desired in terms of values obtained under similar circumstances from normal individuals of the same age and sex.

It is perhaps even more desirable to adjust the hemoglobin content to an arbitrary standard red cell count, usually 5,000,000. To illustrate, in their standardization of the copper sulfate–specific gravity method for hemoglobin and other blood proteins (p. 607), Phillips, Van Slyke, *et al.*, found the average oxygen capacity of 20 normal adult men to be 21.6 vols. per cent, corresponding to a hemoglobin content of 15.9 g. per cent. If the average red cell count under these conditions is 5.4 million, an acceptable value, the hemoglobin content on the basis of 5.0 million red cells will be 15.9 × 5.0/5.4, or 14.7 g. per cent.[175] In this connection, it is further of interest to note that while adult females have an apparent average hemoglobin content lower by about 1.0 g. per cent than adult males, this difference largely disappears if results are corrected for the variation in red cell count as described above.[176]

1. "Acid Hematin" Method of Cohen and Smith:[177] ***Principle.*** Blood is treated with dilute hydrochloric acid to produce a brown color ("acid hematin"). This is then compared with the color produced by similar treatment of a blood of known hemoglobin content.

Procedure.[178] **Measure 0.05 ml. of blood from a freely flowing source into an accurate micropipet, calibrated "to contain." If the blood appears to be low in hemoglobin, it is advisable to use a double sample. Wipe off excess blood from the outside of the pipet, and blow the blood from the pipet into**

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[175] Haden's value on a 5.0 million red cell basis is 15.6 g. per cent (*J. Am. Med. Assoc.*, **79**, 1496 (1922)). Dr. Ralph G. Stillman (personal communication) has summarized literature reports relative to 11 large cities throughout the world and finds an average value of 14.7 g. per cent computed in terms of a 5.0 million red cell count.

[176] Haden: *loc. cit.*

[177] Cohen and Smith: *J. Biol. Chem.*, **39**, 489 (1919).

[178] Reagents Required: *Dilute Hydrochloric Acid.* Dilute 10 ml. of concentrated hydrochloric acid to 1 liter with water and mix. This solution is approximately 0.1 normal; it need not be standardized.

Standard "Acid Hematin" Solution. Obtain a large sample of normal human blood and determine its hemoglobin content by either gasometric methods (Chapter 24) or the iron method of Wong (p. 617). On the basis of the hemoglobin content, dilute the blood with 0.1 N hydrochloric acid in a volumetric flask so that the resultant hemoglobin concentration is 3 per cent. Thus if the hemoglobin content is 14.2 g. per cent, dilute 21.1 ml. $\left(\text{i.e., } \frac{100 \times 3}{14.2}\right)$ to 100 ml. with 0.1 N acid. Mix well and store in the refrigerator. This stock standard will keep for three months if kept cold and away from light. It must be well-mixed just before using, because the brown color is due to a colloidal suspension of hemin, which will settle out on standing. From the stock solution, the dilute standard is prepared fresh every week by diluting 5 ml. to 200 ml. in a volumetric flask with 0.1 N acid. Keep cold and away from light, and mix well before using. This dilute standard is an "acid hematin" solution equivalent to 0.075 g. per cent hemoglobin (i.e., to 15 g. per cent blood hemoglobin at a 1:200 dilu-

10 ml. of dilute hydrochloric acid. Rinse the pipet thoroughly by sucking up the acid and blowing back several times. Allow to stand at room temperature for at least 1 hour to complete color development, or, if speed is essential, place in hot tap water (50 to 55° C.) for 10 minutes.[179] Read in the colorimeter or photometer against the standard "acid hematin" solution. For photometric measurement, set the photometer to zero density with water or dilute acid as a blank, at 520 mμ.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.075 \times \frac{100}{0.05} \times \frac{10}{100} = \text{grams hemoglobin per 100 ml. blood}$$

In this calculation, the 0.075 represents the hemoglobin content of the standard, in g. per 100 ml.; the 0.05 is the volume of blood taken, and the 10 (more exactly 10.05) represents the volume to which the blood is diluted. If smaller or larger amounts of blood are used, at the same or different dilution volumes, change the calculation accordingly. A blue glass or gelatin filter over the eyepiece of the colorimeter improves the precision of color match.

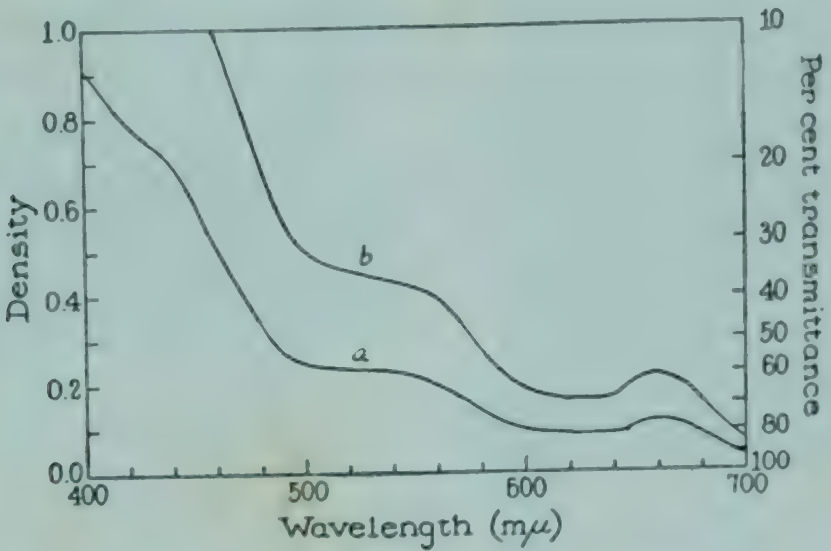

FIG. 163. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN "ACID HEMATIN" METHOD FOR HEMOGLOBIN.

For (*a*) 7.5 g. per cent hemoglobin, and (*b*) 15 g. per cent hemoglobin, each at a 1:200 dilution. Solution depth, 1 cm.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.075 \times \frac{100}{0.05} \times \frac{10}{100} = \text{grams hemoglobin per 100 ml. blood}$$

The "acid hematin" color, which is really a colloidal suspension of hemin (see Chapter 22), has no characteristic peak light absorption in the visible spectrum (Fig. 163).

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tion). For a method of preparing standard "acid hematin" solutions from crystalline hemin, see Elvehjem: *J. Biol. Chem.*, **93**, 203 (1931).

[179] If both unknown and standard are treated with one-tenth volume of 10 per cent sodium hydroxide at this point, after color development and prior to reading, the procedure becomes the "alkali hematin" method of Wu (*J. Biochemistry* (Japan), **2**, 173 (1922)), claimed to be superior to the "acid hematin" method. See, however, Ponder: *J. Biol. Chem.*, **144**, 339 (1942). Conditions of color measurement and calculations are the same as for the procedure described in the text.

The choice of filter depends largely upon the sensitivity desired. At 520 mμ, and in a 1-cm. cuvette, the standard (equivalent to 15 g. per cent hemoglobin at a dilution of 1:200) has a density of approximately 0.500, and agreement with Beer's law is excellent over the entire range of hemoglobin content apt to be encountered.

This method has the advantage of a relatively permanent standard, in terms of which each unknown may be measured. It will probably be found that for a given photometer and filter or wavelength setting the density of the standard will be constant and reproducible from day to day if mechanical or other changes do not occur in the photometer and if measurements are made under uniform conditions. In this event, the established density of the standard may be used in the photometric calculations without the necessity of reading the standard at each analysis. To eliminate error due to any changes in calibration, however, it is advisable to check the standard reading at intervals.

Interpretation. The hemoglobin content as determined by either the "acid hematin" or "alkali hematin" methods represents *total hemoglobin*, i.e., the methods do not distinguish between hemoglobin itself and such nonfunctional derivatives as methemoglobin, carbon monoxide hemoglobin, etc. Thus the results are apt to be slightly high as compared to oxygen capacity measurements, particularly in the case of city dwellers and chronic tobacco smokers, whose blood may contain up to 5 per cent of its total hemoglobin as carbon monoxide hemoglobin. The "acid hematin" method as described is not applicable to the blood of species possessing nucleated erythrocytes (i.e., birds) because of the turbidity caused by the nuclei;[180] the "alkali hematin" method does not have this disadvantage.

In normal individuals, at birth the blood hemoglobin content may be over 21 g. per 100 ml. of blood, dropping to about half this value, or around 11 to 12 g. per cent, during the first six months after birth, and remaining at approximately this level up to about three years of age, after which there is a gradual increase until the age of 16 or so is reached. At this point, the hemoglobin content reaches a level which tends to be maintained throughout life; for males this level lies between roughly 14.5 and 16.0 g. per cent; for females, 13.5 and 15.0 g. per cent. As indicated on p. 613, the sex difference is due largely to differences in red cell count. This factor does not appear to explain the effect of age on hemoglobin content.

Aside from age and sex, other factors which influence the normal hemoglobin content include climate, altitude, exercise, menstruation, diurnal variation, and in general any factor which influences the red cell count or plasma volume. A *decreased* hemoglobin content is found in the various anemias, in pregnancy, after moderately severe or chronic hemorrhage, and following the excessive intake of fluids. An *increased* hemoglobin content, usually accompanied by an increase in red cell count (*polycythemia*) is found in hemoconcentration due to either shock or dehydration, in anoxia caused by either low oxygen pressure (high altitudes) or failure to properly oxygenate blood because of cardiac or pulmonary involvement, and experimentally after the administration of cobalt salts.

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[180] For adaptation of the "acid hematin" method to chicken blood, see Schultze and Elvehjem: *J. Biol. Chem.*, **105**, 253 (1934).

2. *Method of Newcomer:*[181] *Principle.* The blood is treated with dilute hydrochloric acid to produce the brown "acid hematin" color described for the previous procedure. This is then matched in a colorimeter against a brown glass plate which has been spectrophotometrically standardized to correspond to the color obtained from a known amount of hemoglobin. This method is relatively little used, having been replaced by the various photometric procedures.

3. *Direct Photometric Method: Principle.* Blood is diluted in weakly alkaline solution. The color intensity is then measured in a photometer at 540 mμ, and the hemoglobin content estimated from the established reading of a blood of known hemoglobin content under similar conditions. In the procedure described here, very dilute ammonia solution is used for diluting the blood; 0.1 per cent sodium carbonate solution has also been proposed for this purpose.[182] If the diluted blood is treated before reading with a source of carbon monoxide gas, such as ordinary illuminating gas, the procedure becomes the more exact carbon monoxide hemoglobin method of Palmer.[183]

Procedure. **Collect 0.02 ml. of blood in an accurate micropipet calibrated "to contain." Wipe off excess blood from the outside of the pipet, and transfer the blood to 5 ml. of dilute ammonium hydroxide solution[184] in a test tube. Rinse the pipet with the solution by filling and emptying several times. Mix, transfer to a suitable container if necessary, and read in the photometer at 540 mμ. Set the photometer to zero density with a blank of the dilute ammonia solution alone. For the Palmer procedure, bubble illuminating gas through the solution (in the hood) for about 30 seconds before reading in the photometer at 540 mμ.**

CALCULATION. Density of Unknown × *F* = grams hemoglobin per 100 ml. blood. *F* is a factor established by running the above-described procedure on a blood of known hemoglobin content. Obtain a sample of normal human blood and determine its hemoglobin content by either the gasometric method (Chapter 24) or, if gasometric equipment is not available, by the method of Wong described on p. 617. Treat duplicate or triplicate portions of this standard blood by the procedure described above. From the determined density and the hemoglobin content, calculate *F* as follows:

$$F = \frac{\text{Hemoglobin content, in g. per cent}}{\text{Density}}$$

Once established for a given photometer and filter or wavelength setting, the value of *F* will ordinarily be constant unless mechanical or other changes occur in the photometer. Checking the calibration at intervals will eliminate errors due to change in the value of *F*. In general, the calibration factor for one instrument and filter is not applicable to another instrument or filter, even of the same make.

At 540 mμ, and in a 1-cm. cuvette, a blood with 15.0 g. per cent hemoglobin has a density of approximately 0.500 when simply diluted as described; after carbon monoxide treatment, the density is approximately 0.550. For spectrophotometric data, see Fig. 110, p. 473. Beer's law is valid over the entire range of hemoglobin content apt to be encountered. For measurement using deeper cuvettes, measure the blood into 10 ml. of ammonia solution instead of 5 ml., establishing *F* on the same basis.

Interpretation. In the absence of significant amounts of abnormal blood pigments, both the simple dilution procedure and the Palmer method are known to give satisfactory results. The Palmer method is more accurate, since it is free from any error due to the presence of carbon monoxide hemoglobin in the blood as drawn. The chief disadvantage of the Palmer method in the past has been the difficulties associated with the preparation and maintenance of a suitable standard; this is eliminated by photometric calibration. The illuminating gas must of course be free from substances other than carbon monoxide which are capable of reacting with hemoglobin. If the blood contains significant amounts of methemoglobin, results will be in error. For the photometric determination of methemoglobin and total hemoglobin in the presence of methemoglobin, see the method of Evelyn and Malloy (p. 619). For other aspects of interpretation, see previous methods.

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[181] Newcomer: *J. Biol. Chem.*, **37**, 465 (1919): **55**, 569 (1923).

[182] Sanford, Sheard, and Osterberg: *Am. J. Clin. Path.*, **3**, 405 (1933).

[183] Palmer: *J. Biol. Chem.*, **33**, 119 (1918).

[184] Dilute 4 ml. of concentrated ammonium hydroxide to 1 liter with water and mix. Stable indefinitely.

4. Method of Wong.[185] ***Principle.*** The iron is detached from the hemoglobin molecule by treatment with concentrated sulfuric acid in the presence of potassium persulfate, without heating. After removal of the proteins by tungstic acid, the iron in the filtrate is determined colorimetrically. From the total iron content, the hemoglobin content is readily obtained, since hemoglobin contains 0.34 per cent of iron, and only about 1 to 2 per cent or less of the total blood iron is nonhemoglobin iron.

Procedure.[186] **With an Ostwald or micropipet, accurately transfer 0.5 ml. of well-mixed oxalated whole blood to a 50-ml. volumetric flask. Add 2 ml. of iron-free concentrated sulfuric acid. Mix by whirling one to two minutes. Add 2 ml. of saturated potassium persulfate solution. Mix and dilute to about 25 ml. with water. Add 2 ml. of 10 per cent sodium tungstate solution. Mix. Cool to room temperature under the tap, and dilute to volume with water. Stopper and mix by inversion. Filter through a dry paper, collecting the filtrate in a dry flask. Prepare a standard in a second 50-ml. volumetric flask by adding to about 25 ml. of water in the flask the following: 2 ml. of concentrated sulfuric acid, 2 ml. of saturated potassium persulfate solution, and 2.5 ml. of standard iron solution containing 0.1 mg.**

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[185] Wong: *J. Biol. Chem.*, **77**, 409 (1928). Hanzal (*Proc. Soc. Exptl. Biol. Med.*, **30**, 846 (1933) uses sulfuric acid and hydrogen peroxide to effect complete oxidation of organic matter, followed by the thioglycollic acid colorimetric procedure described as an alternative method in the text. For a modification of the Wong method, claimed to be superior, see Ponder: *J. Biol. Chem.*, **144**, 333 (1942).

[186] Reagents Required: *Concentrated Sulfuric Acid*, iron-free.

Saturated Potassium Persulfate Solution. Shake 7 to 8 g. of reagent-grade iron-free potassium persulfate with 100 ml. of water in a glass-stoppered bottle. The undissolved excess settles to the bottom and compensates for loss by decomposition. Shake briefly before using. Keep in the refrigerator.

10 Per Cent Sodium Tungstate Solution. Dissolve 100 g. of reagent-grade iron-free sodium tungstate in water and dilute to 1 liter. Of the various reagents, it is most important that the tungstate be iron-free, since it is the only reagent whose iron content is not corrected for in photometric measurement by the use of a blank as described in the text.

Standard Iron Solution. Dissolve 0.702 g. of reagent-grade crystalline ferrous ammonium sulfate ("Mohr's salt") in 100 ml. of water. Add 5 ml. of concentrated sulfuric acid, warm slightly, and add concentrated potassium permanganate solution drop by drop until 1 drop produces a permanent color. Transfer to a 1-liter volumetric flask with rinsings, dilute to the mark, and mix. This solution contains 0.1 mg. of ferric iron per ml., and is stable indefinitely.

3 N Potassium Thiocyanate Solution. Dissolve 146 g. of reagent-grade potassium thiocyanate in water and dilute to 500 ml. Filter if turbid. Add 20 ml. of pure acetone to improve the keeping quality. Deterioration will be evidenced by the rapid formation of a yellow color in the blank test described in the procedure.

Thioglycollic Acid. Obtainable from Eastman Kodak Co., Rochester, New York.

Concentrated Ammonium Hydroxide, iron-free.

of ferric iron per ml. Cool to room temperature, dilute with water to the mark, and mix. For photometric measurement, prepare a blank similar to the standard except that the standard iron solution is omitted.

Measure 10 ml. of unknown filtrate, standard, and blank if necessary, into separate test tubes. To each add 0.5 ml. of saturated persulfate solution followed by 2 ml. of 3 N potassium thiocyanate solution. Mix by inversion and read in the colorimeter or photometer within the next 30 minutes. For photometric measurement, set the photometer to zero density with the blank, at 480 mμ.

An alternative procedure for color development using thioglycollic acid is as follows: To the 10-ml. portions in small flasks or test tubes as described above, add 0.1 ml. of thioglycollic acid,[187] and 2 ml. of concentrated ammonium hydroxide. Mix well, cool to room temperature by placing in cold water, and read in the colorimeter or photometer at any time within the next hour. Just before reading, shake again; the color may fade on prolonged standing but it can be immediately restored by shaking in air. For photometric measurement, set the photometer to zero density with the blank, at 540 mμ.

CALCULATION. *For colorimetric measurement,* using either the thiocyanate or thioglycollic acid procedure:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.25 \times \frac{100}{0.5} \times \frac{1}{3.4} = \text{grams hemoglobin per 100 ml. blood}$$

The value 1/3.4 represents the fact that 1 g. of hemoglobin contains 3.4 mg. of iron. If this factor is omitted in the calculations, the result gives *mg. of total iron* per 100 ml. of blood. Under ordinary circumstances, less than 2 per cent of the total blood iron is from sources other than hemoglobin; this nonhemoglobin iron is therefore neglected in the calculation of hemoglobin content, or a suitable correction may be made.

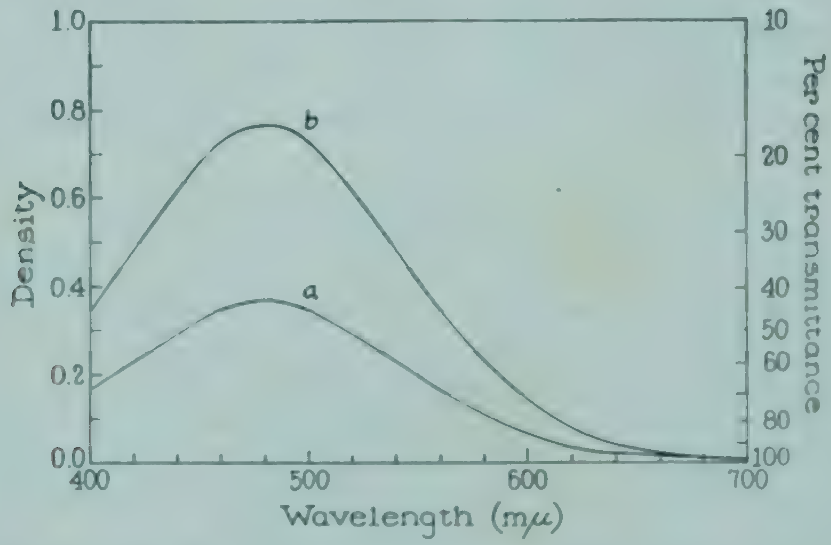

FIG. 164. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN WONG METHOD FOR IRON AND HEMOGLOBIN.

For standards containing (*a*) 1.25 mg., and (*b*) 2.5 mg. iron in 50 ml. Solution depth, 1 cm.

For photometric measurement, using either procedure:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.25 \times \frac{100}{0.5} \times \frac{1}{3.4} = \text{grams hemoglobin per 100 ml. blood}$$

The thiocyanate color has maximum absorption at about 480 mμ. (Fig. 164). At this wavelength, and in a 1-cm. cuvette, the density of the standard described is approximately 0.750. Since the standard corresponds to a blood iron content of 50 mg. per cent, equivalent to 14.7 g. per cent hemoglobin, all values of hemoglobin ordinarily encountered may be satisfactorily determined under these conditions. With deeper cuvettes, use a 5-ml. aliquot of filtrate instead of 10 ml., add 5 ml. of the reagent blank solution used to set the photometer to zero density, develop and read the color as described, and multiply the results by 2. If a filter at 480 mμ is not available, equally satisfactory results at about 40 per cent lower scale reading may be obtained at 420 or 520 mμ.

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[187] Burmester (*J. Biol. Chem.*, **105**, 189 (1934)) adds sodium sulfite solution prior to adding the thioglycollic acid, to reduce the persulfate. This does not appear to be necessary.

The thioglycollic acid color has maximum absorption at 540 mμ. As compared to the thiocyanate color, the density of the standard under similar conditions is considerably less, being approximately 0.240 at peak absorption. This is not too low for accurate photometric measurement, however, and the color is superior in many respects to the thiocyanate color for analytical purposes. Excellent agreement with Beer's law is found up to double the standard concentration at almost any wavelength between 400 to 580 mμ. The only disadvantage appears to be a tendency of the color to fade on standing; as already mentioned, restoration to the original value is easily accomplished by brief shaking in air.

Interpretation. This method is recommended for standardization of other hemoglobin procedures in the absence of facilities for determining oxygen capacity. For other aspects of interpretation, see previous methods.

5. Photometric Determination of Methemoglobin and Total Hemoglobin (Method of Evelyn and Malloy):[188] ***Principle.*** Methemoglobin has a characteristic light absorption at 635 mμ (see Fig. 110, p. 473); this absorption is abolished in the presence of cyanide, which converts methemoglobin to cyanomethemoglobin. The difference in light absorption at 635 mμ before and after adding cyanide is a measure of the methemoglobin present. Total hemoglobin is determined by converting all the hemoglobin present to cyanomethemoglobin and measuring the light absorption at 540 mμ. Evelyn and Malloy also describe a procedure for measuring sulfhemoglobin with the same sample; this is omitted here because of the uncertainty associated with standardization. For details, see original article.

Procedure.[189] **Transfer 0.1 ml. of capillary or well-mixed oxalated venous blood to a test tube containing 10 ml. of M/60 phosphate buffer at pH 6.6. Mix and allow to stand 5 minutes.**

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[188] Evelyn and Malloy: *J. Biol. Chem.*, **126**, 655 (1938).

[189] Reagents Required: *M/15 Phosphate Buffer, pH 6.6.* Dissolve 9 g. of $Na_2HPO_4 \cdot 12H_2O$ and 5.7 g. of anhydrous KH_2PO_4 in water and dilute to 1 liter.

M/60 Phosphate Buffer, pH 6.6. To 1 volume of M/15 phosphate buffer, pH 6.6, add 3 volumes of water, and mix.

20 Per Cent Potassium Ferricyanide. Dissolve 20 g. of reagent-grade potassium ferricyanide in water with the aid of heat, cool, and dilute to 100 ml. Dispense from a dropper bottle which delivers about 25 drops per ml. To prepare the 5 per cent solution, dilute 1 volume of the 20 per cent solution with 3 volumes of water.

10 Per Cent Sodium Cyanide. (*Poisonous!*) To 5 g. of reagent-grade sodium cyanide in a beaker, add 50 ml. of water, and stir to dissolve. Transfer to a dropping bottle for dispensing, as above.

Neutralized Sodium Cyanide. (*Poisonous!*) Mix 1 volume of 10 per cent sodium cyanide solution and 1 volume of 12 per cent acetic acid (12 ml. of glacial acetic acid diluted to 100 ml. with water). Add the acid to the cyanide (not the reverse) quickly with mixing, *in the hood.* Make only as much as will be used up within an hour, transferring it to a small dropping bottle for dispensing. Avoid exposure to vapors from the solution.

Concentrated Ammonium Hydroxide, in a dropping bottle as above.

(a) METHEMOGLOBIN. Determine the density of the solution in a photometer at 635 mμ, setting the photometer to zero density with water. This is reading D_1. Add 1 drop of neutralized sodium cyanide to the entire 10-ml. sample, mix, allow to stand 2 minutes, and make a second reading, D_2, under the same conditions as the first reading. The difference between D_1 and D_2 is the measure of the methemoglobin content which is calculated as described below. Any slight turbidity present is immaterial, since it is the same in both readings.

(b) TOTAL HEMOGLOBIN. Use either the sample treated with cyanide as just described, or the original 1:101 dilution of the blood if total hemoglobin alone is to be determined. Add 1 drop of concentrated ammonium hydroxide to the entire 10-ml. sample to clear it, mix, and transfer a 2-ml. portion to a test tube containing 8 ml. of M/15 phosphate buffer at pH 6.6 and 1 drop of 20 per cent potassium ferricyanide. Mix and allow to stand 2 minutes to convert hemoglobin to methemoglobin. Add 1 drop of 10 per cent sodium cyanide, mix, and again allow to stand 2 minutes for the formation of cyanomethemoglobin. Determine the density in the photometer at 540 mμ, setting the photometer to zero density with a blank consisting of 10 ml. of M/15 phosphate buffer plus 1 drop each of the ferricyanide and the sodium cyanide solutions. Let this reading be D_3.

CALCULATION. (*a*) *Methemoglobin.*

$$(D_1 - D_2) \times F_M = \text{grams methemoglobin per 100 ml. blood}$$

where F_M is a factor expressing the relationship between a known amount of methemoglobin and the change in density at 635 mμ after adding cyanide. This factor is established as described below.

(*b*) *Total Hemoglobin.*

$$D_3 \times F_T = \text{grams total hemoglobin per 100 ml. blood}$$

where F_T is the calibration factor for hemoglobin as cyanomethemoglobin, determined as described below.

DETERMINATION OF CALIBRATION FACTORS.[190] Obtain a sample of normal human blood and determine its hemoglobin content by either the gasometric method (Chapter 24) or the iron method of Wong (p. 617). Of the well-mixed blood, transfer 0.1 ml. to a tube containing 9.9 ml. of M/60 phosphate buffer at pH 6.6 and 0.1 ml. of 5 per cent potassium ferricyanide solution. Mix, allow to stand 2 minutes, and then determine the density (= D_1) in the photometer at 635 mμ, setting to zero density with a blank consisting of 10 ml. of the phosphate buffer plus 0.1 ml. of the ferricyanide. After the reading has been made, add 1 drop of neutralized sodium cyanide solution to both the entire sample and blank, mix, and allow to stand 2 minutes, and again determine the density (= D_2) against the blank, at the same wavelength. The factor F_M is calculated as follows:

$$\frac{\text{Hemoglobin content of blood, in g. per cent}}{(D_1 - D_2)} = F_M$$

To illustrate: in a 1-cm. cuvette under the conditions described, a blood of 15.2 g. per cent hemoglobin content gave a D_1 reading of 0.357 and a D_2 reading of 0.055

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[190] Evelyn and Malloy do not describe the obtaining of calibration factors, but give instead the numerical values for the various factors as established by them for the Evelyn photoelectric colorimeter. The factors given by Evelyn and Malloy may be employed under their conditions, but independent verification is suggested.

The factor is therefore

$$\frac{15.2}{(0.357 - 0.055)} = \frac{15.2}{0.302} = 50.4$$

To obtain F_T, dilute a 2-ml. portion of both the ferricyanide-cyanide treated standard and blank to 10 ml. with water and mix. Set the photometer to zero density at 540 mμ with the blank, and determine the density of the standard ($= D_3$).

$$F_T = \frac{\text{Hemoglobin content of standard, in g. per cent}}{D_3}$$

To illustrate: the D_3 reading for the blood containing 15.2 g. per cent hemoglobin was 0.211. Therefore $F_T = 15.2/0.211 = 72.0$.

Once they have been carefully established for a particular photometer and filter or wavelength setting, these factors should be valid indefinitely unless mechanical or other changes occur in the photometer. Checking at intervals will eliminate errors due to such changes.

Interpretation. The limit of precision of both the methemoglobin and total hemoglobin methods as described here is about 0.1–0.2 g. per cent. Using these methods with a Beckman spectrophotometer, in a study of 14 normal young men daily over a four-day period it was not possible to find even 0.1 g. per cent of methemoglobin in any except three random samples. Paul and Kemp,[191] using a similar procedure but at a lower dilution of blood (and therefore possibly increased sensitivity), claim that small amounts of methemoglobin (0.03 to 0.13 g. per cent) are regularly present in normal blood. Increased methemoglobin content (*methemoglobinemia*) is associated with the administration of a variety of drugs, such as nitrites, aniline and derivatives, sulfanilamide, acetanilide, etc. When the drug is discontinued, the methemoglobinemia begins to decrease; it is thought that methemoglobin can be converted back to hemoglobin in the red cell, hence any evident methemoglobinemia presumably represents a balance between rate of production and rate of reconversion to hemoglobin.[192]

The cyanomethemoglobin method for total hemoglobin is considered to be one of the most accurate of the colorimetric methods. Results will be slightly in error in the few instances where the relatively rare pigment *sulfhemoglobin* is present; Evelyn and Malloy (*loc. cit.*) discuss this possibility of error and describe a correction for it. For further aspects of interpretation, see previous methods.

Other Methods. Hemoglobin may be quickly and accurately determined, along with the total plasma protein, by the copper sulfate–specific gravity method (p. 607). The use of pyridine hemochromogen has also been advocated as a basis for colorimetric or photometric measurement.[193] Among the advantages claimed is that the method may be accurately standardized using pure hemin. Ultramicromethods based on the benzidine color test for blood (see Chapter 22) have been described by Wu[194] and by Bing and Baker.[195] For the colorimetric determination of carbon monoxide hemoglobin, see p. 495. Gasometric procedures for the determination of hemoglobin, carbon monoxide hemoglobin, and methemoglobin are described in Chapter 24.

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[191] Paul and Kemp: *Proc. Soc. Exptl. Biol. Med.*, **56**, 55 (1944).

[192] Cox and Wendel: *J. Biol. Chem.*, **143**, 331 (1942).

[193] Rimington: *Brit. Med. J.*, **1**, 177 (1942); Flink and Watson: *J. Biol. Chem.*, **146**, 171 (1942).

DETERMINATION OF LACTIC ACID

Introduction. Blood lactic acid is ordinarily determined by conversion to acetaldehyde, which is then measured by titrimetric or colorimetric methods. A gasometric procedure based upon oxidation with permanganate to produce carbon dioxide has also been described.[196] Of the various methods, the colorimetric method described here is by far the most sensitive, being readily applicable to 0.1 ml. or less of blood. In obtaining blood for lactic acid determination, precautions must be observed against the conversion of blood glucose to lactic acid on standing (*glycolysis*). The use of fluoride as anticoagulant (see p. 541) will prevent glycolysis; if oxalate or heparin are used, the protein-free filtrate should be prepared as soon as possible after drawing the blood.[197]

1. Method of Barker and Summerson:[198] ***Principle.*** The glucose and other interfering material of the protein-free blood filtrate is removed by the Van Slyke-Salkowski method of treatment with copper sulfate and calcium hydroxide. An aliquot of the resulting solution is heated with concentrated sulfuric acid to convert lactic acid to acetaldehyde, which is then determined colorimetrically by reaction with *p*-hydroxydiphenyl in the presence of copper ions.

Procedure.[199] **Deproteinize the blood sample (whole blood, plasma) with either tungstic acid (p. 543), trichloroacetic acid (p. 631), or zinc hydroxide**

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[194] Wu: *J. Biochemistry* (Japan), **2**, 189 (1922).

[195] Bing and Baker: *J. Biol. Chem.*, **92**, 589 (1931). See also McFarlane and Hamilton: *Biochem, J.*, **26**, 1050 (1932).

[196] Avery and Hastings: *J. Biol. Chem.*, **94**, 273 (1931).

[197] Friedemann and Haugen (*J. Biol. Chem.*, **144**, 67 (1942)) describe a procedure in which the drawn blood is ejected directly from the syringe into the protein-precipitating fluid.

[198] Barker and Summerson: *J. Biol. Chem.*, **138**, 535 (1941).

[199] Reagents Required: *20 Per Cent Copper Sulfate.* Dissolve 400 g. of $CuSO_4 \cdot 5H_2O$ in about 1 liter of water with the aid of heat, cool, dilute to 2 liters, and mix. Stable indefinitely.

4 Per Cent Copper Sulfate. Dilute 1 volume of 20 per cent copper sulfate solution to 5 volumes with water and mix. Store in a bottle fitted with a stopper carrying a 1-ml. pipet which delivers approximately 20 drops per ml. If this is done, 1 drop may be used instead of the 0.05-ml. portion specified in the text.

Calcium Hydroxide, Powder. Either the U.S.P. or C.P. grades are satisfactory. It is conveniently dispensed with a spoon spatula known to hold approximately 1 g., since exact measurement is unimportant.

Sulfuric Acid, Concentrated. Reagent-grade iron-free sulfuric acid is satisfactory. It is dispensed from a buret, suitably protected against absorption of atmospheric moisture. The buret stopcock is cleaned thoroughly of grease and lubricated with a little of the acid itself. In delivering, precautions should be taken against error due to the slow drainage of the viscous acid. According to Russell (*J. Biol. Chem.*, **156**, 463 (1944)), nitrates and nitrites in the acid will interfere; only grades with low nitrate content are selected, and if a particular lot shows poor color development it is discarded.

p-Hydroxydiphenyl Reagent. Dissolve 1.5 g. of *p*-hydroxydiphenyl (obtainable from Eastman Kodak Co., Rochester, N.Y.) in 10 ml. of 5 per cent sodium hydroxide solution, plus a little water, by warming and stirring, and dilute to 100 ml. with water. Store in a brown bottle fitted with a stopper and pipet capable of delivering 20 drops per ml. If this is done, 2 drops may be used instead of the 0.1-ml. portion specified in the text. The reagent is stable for many months; deterioration is evidenced by high blank readings.

Standard Lactic Acid Solution. This is prepared preferably from lithium lactate, which is

(p. 572) at a 1:10 dilution. Transfer 2 ml. of the protein-free filtrate, representing 0.2 ml. of blood, to a centrifuge tube graduated at 10 ml. In a second similar tube place 5 ml. of standard lactic acid solution, containing 0.01 mg. of lactic acid per ml. In a third tube place a little water; this is a blank, and serves to control the small amount of color yielded by the reagents alone. To each tube add 1 ml. of 20 per cent copper sulfate solution and dilute to the 10-ml. mark with water. Add 1 g. of powdered calcium hydroxide to each tube, stopper,[200] and shake vigorously until the solids are uniformly dispersed. Allow to stand for one-half hour, repeating the shaking at least once in the interim. Centrifuge down the precipitate, and transfer duplicate 1-ml. portions of the supernatant from each tube to thoroughly clean and dry test tubes having an internal diameter of 18 to 23 mm.[201] To each tube add 0.05 ml. of 4 per cent copper sulfate solution, followed by 6 ml. of concentrated sulfuric acid from a buret. The sulfuric acid should be added drop by drop at first, mixing the contents of the tube well during the addition. The tube contents will become hot; it is not necessary to cool the tube. After the acid has been added to all the tubes, place them upright in boiling water for 5 minutes, then transfer the tubes to cold water (preferably running) and cool to 20° C. or below. When the contents of the tubes are sufficiently cool (but not before) add 0.1 ml. of the *p*-hydroxydiphenyl reagent, drop by drop, to each tube. The reagent precipitates out on entering the concentrated acid; it is dispersed throughout the solution as quickly and uniformly as possible by lateral shaking. When the reagent has been added, place the tubes in a beaker of water at 30° C. and allow to stand for 30 minutes or longer. Redisperse the precipitated reagent at least once during this period. Finally place the tubes in vigorously boiling water for exactly 90 seconds, remove, and cool in cold water to room temperature. Transfer the colored solutions to suitable containers and determine the photometric density at 560 mμ, using water for setting the photometer at zero density.

CALCULATION. Average the duplicate results on the blank to obtain the blank density. Subtract this value from the averages of standard and unknown to obtain their true densities. Since the 1-ml. portion of copper-lime supernatant used for color development contains 0.005 mg. of lactic acid in the case of the standard, and this represents 0.02 ml. of original blood in the unknown (i.e., a dilution of 50), the calculation in this case is as follows:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.005 \times 50 \times 100 = \text{mg. lactic acid per 100 ml. blood}$$

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anhydrous. (For the method of preparing pure lithium lactate, see Chapter 31). For the stock standard, dissolve 0.213 g. of pure dry lithium lactate in about 100 ml. of water in a 1-liter volumetric flask, add about 1 ml. of concentrated sulfuric acid, dilute to the mark with water, and mix. This solution contains 1 mg. of lactic acid in 5 ml., and is stable indefinitely if kept in the refrigerator. To prepare the working standard, dilute 5 ml. of stock standard to 100 ml. in a glass-stoppered volumetric flask with water and mix. This solution contains 0.01 mg. of lactic acid per ml., and is best prepared fresh daily.

[200] Glass-stoppered tubes may be used or, if these are unavailable, cover the mouth of the tube with a small square of "Parafilm," fresh surface down, held in place by the finger tip. "Parafilm" may be obtained from laboratory supply houses. In this and other phases of the procedure, contact of the solutions with the skin must be avoided because of the possibility of contamination with lactic acid from the skin surfaces.

[201] Wide test tubes are specified to facilitate thorough mixing by lateral shaking; this is more important than usual because of the viscosity of the concentrated acid used as solvent. After use, the tubes are best cleaned by simply rinsing in hot tap water, followed by distilled water, and drying by drainage or in an oven. None of the glassware used in this procedure should be cleaned with chromic acid cleaning mixture. Hot soapy water followed by thorough rinsing with distilled water is adequate.

It is recommended that the average blank density be determined separately as described to minimize error due to possible variation in the blank. If desired, a single blank tube may be run and used for setting the photometer to zero density, in which case the average measured densities of standard and unknown are used directly in the calculation. The blank color is ordinarily about 10 per cent of the standard color. In a 1-cm. cuvette, at 560 mμ, the standard described (equivalent to 25 mg. per cent blood lactic acid) has a density of approximately 0.400 (Fig. 165) and up to 60 mg. per cent blood lactic acid may be accurately determined. For higher values, or with deeper cuvettes, use smaller aliquots of filtrate and standard for the copper-lime treatment but keep the final volume at 10 ml. at this stage, using the same amounts of 20 per cent copper sulfate solution and calcium hydroxide as described. Correct the calculations as necessary.

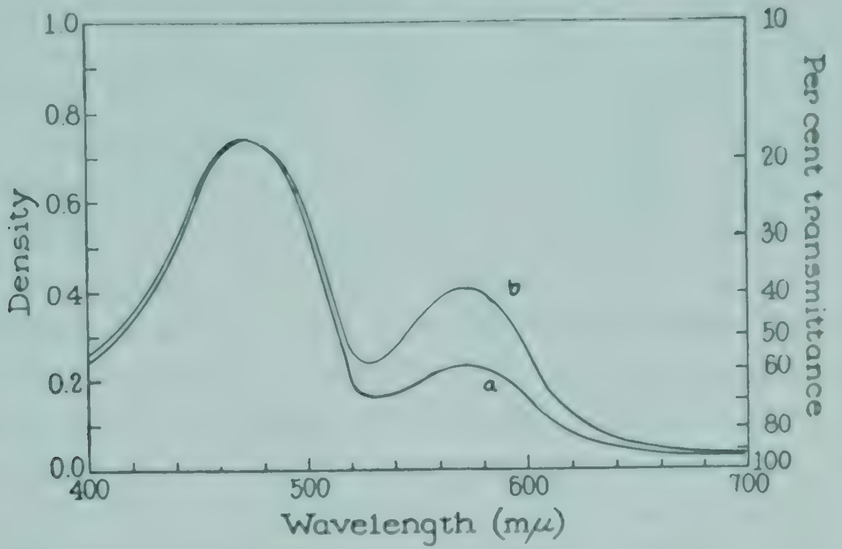

FIG. 165. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN BARKER-SUMMERSON LACTIC ACID METHOD.

For standards containing (*a*) 0.002 mg., and (*b*) 0.004 mg. lactic acid. Solution depth, 1 cm.

Interpretation. Venous blood of normal individuals in the resting state contains 5 to 20 mg. of lactic acid per 100 ml.[202] During severe exercise this may rise to well over 100 mg. per cent, decreasing rapidly during recovery. Pathologically, increased blood lactate content is noted in general whenever there is a deficient supply of oxygen to the tissues (pneumonia, heart failure) or the organism is unable to maintain the normal equilibrium between lactate production and utilization, as after the administration of anesthetics and in liver disease. Usually the presence of excessive amounts of blood lactate is at the expense of equivalent amounts of blood bicarbonate, and an acidosis results. This disappears as the lactate is utilized.

2. *Method of Friedemann and Graeser:*[203] *Principle.* The glucose of blood filtrates is removed with copper sulfate and calcium hydroxide, the lactic acid converted to acetaldehyde, which is then combined with sodium bisulfite. The bound sulfite is determined iodometrically, as in the method for urine (see p. 916).

***Procedure.* To 10 ml. of Folin-Wu or Somogyi filtrate representing 1 ml. of blood add 2 ml. of 10 per cent $CuSO_4$ and 2 ml. of 5 per cent suspension of**

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[202] To convert mg. per cent lactic acid into milliequivalents per liter, divide by 9.0.

[203] Friedemann and Graeser: *J. Biol. Chem.*, **100**, 291 (1933). See also Wendel: *J. Biol. Chem.*, **102**, 47 (1933); Edwards: *ibid.*, **125**, 571 (1938).

$Ca(OH)_2$. Shake at intervals for one-half hour and centrifuge. Of the filtrate 5-ml. duplicates are taken for analysis as in the method for urine (page 916) using 0.002 N iodine. For the blank treat 10 ml. of 0.1 per cent glucose in the same manner as blood filtrate.

Interpretation. See the previous method.

Determination of Lactic Acid in Tissues, Tissue Extracts, Etc. Either of the procedures described above is suitable for the determination of the lactic acid content of various types of biological material. For tissue analysis, precautions must be taken against post-mortem changes in lactic acid content, by prompt freezing in solid carbon dioxide ("dry ice") or by adequate treatment with acid to destroy enzyme systems present. Proteins present may be removed by any of the common methods. For the colorimetric procedure, an aliquot of the protein-free fluid containing 0.02 to 0.10 mg. of lactic acid is treated by the copper-lime procedure described at a volume of 10 ml., and 1 ml. of supernatant is analyzed as described for blood. For the iodometric procedure, the aliquot should contain about 0.5 mg. of lactic acid. This is diluted to 10 ml. and treated as described for blood.

DETERMINATION OF CHLORIDES

Introduction. The chlorides of whole blood are distributed to the extent of about one-third of the total in the red cells and two-thirds in the plasma. Plasma or serum is therefore ordinarily used for analysis; if whole blood were used, variations in red cell content would affect results out of proportion to their clinical significance. In obtaining plasma, excessive amounts of anticoagulants such as oxalate must be avoided, since they influence water and chloride distribution between cells and plasma; heparin does not have this effect. For precise work, where small changes in chloride content are of significance, blood should be collected under oil to minimize changes in carbon dioxide tension, since this also affects chloride distribution between cells and plasma (*chloride shift*, see "Role of the Red Cells," Chapter 24). This precaution is not necessary in routine clinical practice, but it is important that measurements be made carefully, preferably using volumetric flasks for dilution as in the preparation of protein-free filtrates, because of the high chloride content of plasma or serum and the slight variations which are of significance.

Chloride content is commonly expressed in terms of milligrams of sodium chloride per 100 ml. of sample. It is more exact, and preferable, to express chloride concentration in terms of milliequivalents of chloride per liter, since the major functions of chloride in the body are concerned with osmotic pressure regulation and acid-base balance. One milliequivalent of chloride ion corresponds to 35.5 mg., or 58.5 mg. of sodium chloride. A plasma with a chloride concentration of 585 mg. per cent expressed as sodium chloride therefore contains 100 milliequivalents of chloride per liter. The general relationship between these two methods of expressing chloride content is as follows:

$$\text{milliequivalents of chloride per liter} \times 5.85$$
$$= \text{mg. chloride as sodium chloride per 100 ml.}$$

1. Method of Whitehorn.[204] ***Principle.*** The chlorides are precipitated from the blood filtrate by means of silver nitrate in the presence of nitric acid, and the excess of silver titrated with standard thiocyanate solution, using ferric ammonium sulfate as an indicator.

Procedure.[205] **Pipet 10 ml. of the Folin-Wu filtrate into a porcelain dish. Add with a pipet 5 ml. of the standard silver nitrate solution and stir thoroughly. Add about 5 ml. of concentrated nitric acid (sp. gr. 1.42), mix, and let stand for 5 minutes, to permit the flocking out of the silver chloride. Then add with a spatula about 0.3 g. of powdered ferric ammonium sulfate and titrate the excess of silver nitrate with the standard thiocyanate solution until the definite salmon-red (not yellow) color of the ferric thiocyanate persists in spite of vigorous shaking for at least 15 seconds. A microburet should be used in the titration.**

CALCULATION. Subtract the number of ml. of thiocyanate required from 5.00 (= ml. of silver nitrate added) and multiply the result by 100 to obtain the chloride content expressed as mg. of sodium chloride per 100 ml. To express results in terms of milliequivalents of chloride per liter, calculate as above and divide the result by 5.85.

Interpretation. Plasma or serum normally contains from 570 to 620 mg. per 100 ml., expressed as sodium chloride, or 98 to 106 milliequivalents of chloride per liter. The corresponding figures for whole blood are 450 to 500 mg. per cent, or 77 to 86 milliequivalents per liter. Increased plasma chloride is noted in nephritis, and this determination may aid in deciding whether or not salt should be restricted in the diet. Decreased plasma chloride may occur in gastrointestinal disturbances associated with vomiting or diarrhea, in pneumonia, and in Addison's disease. Because of the close association between water and chloride, marked disturbances in water distribution (hydration, dehydration) may not necessarily be accompanied by changes from the normal plasma chloride level.

2. Method of Schales and Schales:[206] ***Principle.*** The sample is titrated with standard mercuric nitrate solution at the proper acidity in the presence of diphenylcarbazone as indicator. Chlorides present react with the added mercuric ions to form soluble but undissociated mercuric chloride. When an excess of mercuric ion has been added, the indicator turns purple. The end point is sharp and relatively stable.

Procedure.[207] **Transfer 2 ml. of Folin-Wu filtrate of plasma or serum (equivalent to 0.2 ml. of original sample) to a small flask, and add 0.06 ml. (4 drops)**

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[204] Whitehorn: *J. Biol. Chem.*, **45**, 449 (1921).

[205] Reagents Required: *Standard Silver Nitrate Solution.* Dissolve 2.905 g. of c.p. silver nitrate in distilled water. Transfer this solution to a liter volumetric flask and make up to the mark with distilled water. Mix thoroughly and preserve in a brown bottle. 1 ml. = 1 mg. NaCl. (It is to be noted that the silver nitrate and nitric acid are not added to the protein-free filtrate simultaneously. To do so may result in the mechanical enclosure of silver nitrate solution within the curds, and hence too high results.)

Standard Thiocyanate Solution. Because thiocyanates are hygroscopic, the standard solution should be prepared volumetrically. Dissolve about 1.7 g. of KCNS or 1.4 g. of NH_4CNS in a liter of water. Titrate against standard silver nitrate solution under the conditions specified under "Procedure," and dilute accurately so that 5 ml. are equivalent to 5 ml. of the silver nitrate solution.

Solid ferric alum is used rather than a solution, in order to insure a very high concentration in the mixture to be titrated. It is powdered to facilitate its solution.

[206] Schales and Schales: *J. Biol. Chem.*, **140**, 879 (1941).

[207] Reagents Required: *Diphenylcarbazone Solution.* Dissolve 100 mg. of s-diphenylcarba-

of diphenylcarbazone indicator solution. Titrate with the standard mercuric nitrate solution, using a microburet capable of being read to 0.01 ml. and delivering small drops. At the end point, the color of the solution changes from light yellow to deep purple.

The plasma or serum may be titrated directly without previous deproteinization. This procedure eliminates errors in the preparation of the filtrate. Transfer 0.2 ml. of sample to a small flask, add 1.8 ml. of water, 0.06 ml. of indicator, and titrate as above. The color of the solution undergoes several changes during the titration, becoming light yellow just before the end point is reached, and changing to pale violet at the end point. Results by the direct titration are slightly higher than when a filtrate is used, possibly because of slight loss of chloride during deproteinization.

CALCULATION. Results for either the protein-free or direct titration are calculated as follows:

$$\text{ml. mercuric nitrate solution used} \times \frac{100}{A} = \text{milliequivalents chloride per liter}$$

where A equals the number of ml. of mercuric nitrate solution required for 2 ml. of standard sodium chloride solution. If A equals 2.00, the calculation simplifies to:

$$\text{ml. mercuric nitrate solution used} \times 50 = \text{milliequivalents of chloride per liter}$$

If results are desired in terms of mg. of sodium chloride per 100 ml., the calculation is as follows:

$$\text{ml. mercuric nitrate solution used} \times \frac{100}{A} \times 5.85 = \text{mg. NaCl per 100 ml.}$$

Interpretation. See the previous method. This method, though not quite so accurate as the iodometric method described subsequently, is relatively simple and is known to give satisfactory results. It has definite advantages over the Whitehorn method. It may be applied to spinal fluid analyses, ordinarily without deproteinization. For the application to urine, see Asper, Schales, and Schales.[208]

3. Method of Sendroy, Modified by Van Slyke and Hiller:[209] ***Principle.*** The plasma or serum is treated with phosphoric acid containing either tungstic acid or

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zone (obtainable from Eastman Kodak Co., Rochester, N.Y.) in 95 per cent alcohol, and dilute to 100 ml. Store in a dark bottle in the cold. Equip the bottle with a rubber-bulb medicine dropper whose tip is adjusted so as to deliver 65 to 70 drops of solution per ml. Prepare fresh solution each month.

Standard Sodium Chloride Solution. Dry some reagent-grade sodium chloride in an oven at 110° to 120° C. overnight. Cool and weigh out 584.5 mg. Dissolve in a little water and transfer with rinsings to a 1-liter volumetric flask. Dilute to the mark with water and mix. This solution is stable indefinitely, and contains 10 milliequivalents of chloride per liter, or 58.45 mg. of sodium chloride per 100 ml. It is used to standardize each new lot of standard mercuric nitrate solution.

Standard Mercuric Nitrate Solution. Place a few hundred ml. of water in a 1-liter volumetric flask and add 20 ml. of 2 N nitric acid. Add 3 g. of reagent-grade mercuric nitrate, dissolve by shaking, dilute to volume with water, and mix. Standardize as follows: transfer 2 ml. of standard sodium chloride solution to a small flask, add 4 drops of diphenylcarbazone solution, and titrate with the mercuric nitrate solution from a microburet as described in the text. The number of ml. of mercuric nitrate solution required equals the value A used in the calculations above. If the strength of the mercuric nitrate solution is adjusted so that A equals 2.00, either by adding more mercuric nitrate or by dilution with water containing 20 ml. of 2 N nitric acid per liter, as the case may be, the calculations are simplified (see text). This standard mercuric nitrate solution is stable indefinitely, and need not be protected from light, so that large amounts may be prepared at one time.

[208] *J. Biol. Chem.*, **168,** 779 (1947).

[209] Sendroy: *J. Biol. Chem.*, **120.** 335, 405, 419 (1937); *ibid.*, **130,** 605 (1939); *ibid.*, **142,** 171 (1942). Van Slyke and Hiller (personal communication).

picric acid, which precipitates the proteins. The mixture is then shaken with an excess of solid silver iodate and filtered. Chlorides present react with the insoluble silver iodate to form insoluble silver chloride and soluble iodate, which passes into the filtrate. On the addition of iodide to the filtrate, the iodate reacts to produce free iodine, which is then titrated with standard thiosulfate.

Procedure.[210] **Transfer 1 ml. of plasma or serum to a 50 ml. Erlenmeyer flask. Add 25 ml. of phosphoric-tungstic acid or phosphoric-picric acid solution and mix. To the mixture add 0.3 to 0.5 g. of silver iodate (measured with sufficient accuracy from a spoon spatula previously found to contain approximately the right amount). Stopper the flask and shake vigorously for 30 seconds. Pour onto a dry 9-cm. folded filter of loose texture, collecting the filtrate in a dry flask.**

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[210] Reagents Required: *Phosphoric-Tungstic Acid Solution.* Transfer 6 g. of reagent-grade chloride-free sodium tungstate to a 1-liter volumetric flask, add water to dissolve, followed by 10 ml. of reagent-grade phosphoric acid, make up to 1 liter with water, and mix. Tungstate may be tested for chloride by dissolving 1 g. in 10 ml. of water, adding 20 ml. of concentrated nitric acid, and filtering into a test tube containing a few ml. of 1 per cent silver nitrate. If the filtrate does not show a precipitate of silver chloride, the tungstate is chloride-free. Tungstate may be freed from chloride by treating a hot concentrated solution with an equal volume of 95 per cent alcohol, filtering off the crystals from the cooled solution, washing with alcohol, and drying in air.

Phosphoric-Picric Acid Solution. This may be used in place of the tungstic acid reagent if chloride-free tungstate is not obtainable; picric acid is less likely to contain chloride. Place 6 ml. of phosphoric acid and 2 g. of picric acid in a 1-liter flask, add water to dissolve, dilute to 1 liter, and mix.

Silver Iodate. This must be free from more soluble iodates. To test, shake 0.5 g. with 25 ml. of phosphoric-tungstic acid solution for 1 minute, filter, and titrate 10 ml. of filtrate with 0.02308 N thiosulfate, after adding iodide, as described above for plasma filtrates. If more than 0.50 ml. of thiosulfate is required at a temperature of 20° C., or 1.00 ml. at 40°, with approximately linear variation at intermediate temperatures, the iodate is not pure enough. To prepare pure silver iodate, dissolve 42.8 g. of potassium iodate in a liter of water and add to it with stirring a liter of silver nitrate solution containing 34 g. of silver nitrate. Filter off the precipitated silver iodate by suction, and wash with water until a 10-ml. portion of the washings when titrated with thiosulfate in the presence of iodide as described above requires no more thiosulfate than corresponds to the solubility of silver iodate as indicated above. Stir up the washed precipitate in 500 ml. of water, suck dry on the Buchner funnel, transfer to a vacuum desiccator to dry thoroughly, and store in a brown bottle.

Sodium Iodide Solution. Dissolve 50 g. of reagent-grade sodium iodide in 50 ml. of water. Store in a brown bottle. This solution must be discarded when a 1-ml. portion added to 10 ml. of phosphoric-tungstic acid solution gives a blue color in the presence of a drop of starch solution. For occasional analyses the dry reagent is more economical. A 0.5-g. portion is used for each analysis. Dispense from a spoon spatula previously found to deliver approximately the right amount.

Standard Sodium Thiosulfate Solution. Prepare a stock thiosulfate solution by dissolving 57.3 g. of crystalline sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$, in water, diluting to 250 ml. in a volumetric flask, and mixing. This solution is stable indefinitely. To prepare the thiosulfate solution used in the titration, transfer 25 ml. of stock solution to a 1-liter volumetric flask, add 1 g. of borax as preservative, dilute to volume with water, and mix. This solution should be 0.02308 N. Standardize as follows: prepare a standard 0.1 N iodate solution by dissolving 3.567 g. of pure potassium iodate in water and diluting to 1 liter. This solution is stable indefinitely. Transfer 5 ml. to a small flask, add about 20 ml. of water and 1 ml. of 1 N acid (sulfuric or hydrochloric). Add 1.5 ml. of sodium iodide solution and titrate immediately with the thiosulfate solution, using starch as indicator. If the volume of thiosulfate required is 21.67 ml., the thiosulfate is 0.02308 N and is used without a factor as described in the procedure above. If some other volume of thiosulfate is required, the factor is obtained by dividing 21.67 by the volume of thiosulfate required. Thus, if 22.40 ml. of thiosulfate were used for titrating the 5 ml. of standard iodate, the factor is 21.67/22.40, or 0.967. This factor is then used in the calculations as described in the procedure. The dilute thiosulfate solution undergoes slow deterioration over a period of months. It should be checked at intervals against the standard iodate solution and a new factor obtained if necessary.

Transfer 10 ml. of the water-clear filtrate to a small flask. Add 1 ml. of sodium iodide solution (or 0.5 g. of solid sodium iodide). Mix carefully with one or two swirls, and titrate the liberated iodine immediately with standard sodium thiosulfate solution. Add the thiosulfate in rapid drops, with careful mixing, until the yellow color of the iodine has almost disappeared. Add 2 drops of starch solution and continue titration until the blue color disappears and the solution is colorless, or, if picric acid was used for deproteinization, only the yellow color of the picric acid remains.

CALCULATION. In this procedure, 1 equivalent of chloride ion leads to the production of 6 equivalents of iodine, requiring 6 equivalents of thiosulfate for titration. Therefore 1 ml. of N thiosulfate represents $\frac{1}{6}$ milliequivalent of chloride. Since the 10 ml. of filtrate titrated represent $\frac{10}{26}$ ml. of sample, the formula for calculation of results in terms of milliequivalents of chloride per liter is as follows:

$$\text{ml. thiosulfate} \times \text{normality} \times \tfrac{1}{6} \times \tfrac{26}{10} \times 1000 = \text{milliequivalents chloride per liter}$$

If the normality of the thiosulfate is exactly 0.02308, the calculation simplies to:

$$\text{ml. thiosulfate} \times 10 = \text{milliequivalents chloride per liter}$$

If the thiosulfate is not exactly 0.02308 N and a factor is used as described under "Standardization," the calculation becomes:

$$\text{ml. thiosulfate} \times \text{factor} \times 10 = \text{milliequivalents chloride per liter}$$

To express results on the basis of milligrams of NaCl per 100 ml., since 1 milliequivalent of chloride corresponds to 58.5 mg. of NaCl, the calculations become:

$$\text{ml. } 0.02308 \text{ N thiosulfate} \times 58.5 = \text{mg. NaCl per 100 ml.}$$

or

$$\text{ml. thiosulfate} \times \text{factor} \times 58.5 = \text{mg. NaCl per 100 ml.}$$

Interpretation. See under the method of Whitehorn above. This method may be used with Folin-Wu filtrates (10 ml. of filtrate made up to 26 ml. with phosphoric-tungstic reagent, treated with iodate, filtered, and titrated exactly as described above, with the same calculations); it is directly applicable on an unchanged basis to the analysis of spinal fluid, gastric contents, urine, etc., and it may be used on a microscale with proportionate decrease in amounts of reagents used, and suitable change in calculations.

4. Determination of Total Chlorides in Blood and Tissues (*Van Slyke*):[211] ***Principle.*** The proteins are oxidized, and the chloride is precipitated, by wet digestion with concentrated nitric acid in the presence of silver nitrate. The excess silver is then titrated with thiocyanate, as described in Whitehorn's method.

DETERMINATION OF PHOSPHORUS

Partition of Blood Phosphorus. Whole blood contains about 40 mg. of total phosphorus per 100 ml., present chiefly as *inorganic phosphate*, *organic acid-soluble phosphate esters*, and *lipide-phosphorus*, with other phosphate-containing compounds possibly present in small amounts. The distribution of phosphorus between cells and plasma is quite uneven;

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[211] Van Slyke: *J. Biol. Chem.*, **58**, 523 (1923); Wilson and Ball: *J. Biol. Chem.*, **79**, 221 (1928).

for example, cells contain much more organic and total phosphate than plasma, while the inorganic phosphate of whole blood is practically entirely in the plasma. For the determination of inorganic phosphate and total acid-soluble phosphorus, protein is precipitated with trichloroacetic acid and the filtrate used. For lipide phosphorus determination, an alcohol-ether extract is obtained. In the determination of total acid-soluble, lipide, and total phosphorus, organic matter is destroyed by digestion with sulfuric acid and 30 per cent hydrogen peroxide. The phosphate-containing solutions thus obtained (as well as the trichloroacetic acid filtrate used for the direct determination of inorganic phosphate) are treated with molybdic acid, whereby phosphomolybdic acid is formed from any inorganic phosphate present. On the addition of suitable reducing agents, phosphomolybdic acid is selectively reduced to yield a deep blue color ("molybdenum blue") which is apparently a mixture of lower oxides of molybdenum. This color is used as a measure of the amount of phosphate present.

Bell and Doisy[212] carried out the reduction with hydroquinone in alkaline solution. Briggs[213] made the color more stable by using an acid solution. Benedict and Theis[214] intensified the color by heating. This procedure is satisfactory where only inorganic phosphate is present, as in serum or plasma filtrates, but cannot be used on whole blood filtrates for example, since any phosphate esters present may be hydrolyzed by the heating in acid and thus lead to high values. Fiske and SubbaRow[215] suggested the use of 1,2,4-aminonaphtholsulfonic acid as reducing agent, at room temperature. This method has been widely used, and is the one described here. Kuttner and Cohen[216] revived the original suggestion of Denigès that stannous chloride be used as reducing agent, and this has likewise found much favor. Stannous chloride has the advantage over aminonaphtholsulfonic acid that the stock reagent is quite stable and the color produced with phosphomolybdate is more intense, thus permitting the estimation of smaller amounts of phosphorus. For example, Shinowara, Jones, and Reinhart[217] have described procedures for phosphate determination, using stannous chloride as reducing agent, which require as little as 0.06 ml. of serum. A disadvantage of stannous chloride is that the color intensity changes continuously with time, so that careful control of the time factor is essential, and deviations from Beer's law are found, usually requiring correction factors. In the authors' experience, the method of Fiske and SubbaRow has proved superior to any other method thus far described.

1. Determination of Inorganic Phosphate** (**Method of Fiske and SubbaRow**[215])**: Principle. The proteins of blood are precipitated with trichloroacetic acid. The protein-free filtrate is treated with an acid molybdate solution, which forms phosphomolybdic acid from any phosphate present. The phosphomolybdic acid is reduced by the addition of 1,2,4-aminonaphtholsulfonic acid reagent, to produce a blue color whose intensity is proportional to the amount of phosphate present.

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[212] Bell and Doisy: *J. Biol. Chem.*, **44**, 45 (1920).

[213] Briggs: *J. Biol. Chem.*, **53**, 13 (1922); **59**, 255 (1924).

[214] Benedict and Theis: *J. Biol. Chem.*, **61**, 63 (1924).

[215] Fiske and SubbaRow: *J. Biol. Chem.*, **66**, 375 (1925).

[216] Kuttner and Cohen: *J. Biol. Chem.*, **75**, 517 (1927); also Kuttner and Lichtenstein: *J. Biol. Chem.*, **86**, 671 (1930).

[217] Shinowara, Jones, and Reinhart: *J. Biol. Chem.*, **142**, 921 (1942).

Procedure.[218] **To 8 ml. of 10 per cent trichloroacetic acid solution in a small flask, add slowly, with mixing, 2 ml. of whole blood, serum, or plasma. Stopper, shake, and filter through a low-ash filter paper. Transfer 5 ml. of filtrate to a cylinder or other container graduated at 10 ml., and add 1 ml. of the Molybdate II reagent. Mix. Add 0.4 ml. of aminonaphtholsulfonic acid reagent, and again mix. Dilute to the mark, mix, and allow to stand 5 minutes.**

For colorimetric measurement, compare in the colorimeter against a standard prepared at the same time, as follows: Transfer 5 ml. of standard phosphate solution, containing 0.4 mg. of phosphorus, to a 100-ml. volumetric flask, and add from a graduate 50 ml. of water. Add 10 ml. of Molybdate I (*not* Molybdate II), mix, and add 4 ml. of aminonaphtholsulfonic acid reagent. Dilute with water to the 100 ml. mark, mix, and allow to stand 5 minutes. Compare the standard against itself in the colorimeter before reading the unknown. If the color of the unknown is particularly strong, repeat the reading of the unknown a few minutes later, to be sure that maximal color development has taken place.

For photometric measurement, transfer a portion of the colored solution to a suitable container and read in the photometer at 660 to 720 mμ (see Fig. 166). Set the photometer to zero density with a blank prepared by treating 5 ml. of 10 per cent trichloroacetic acid with 1 ml. of Molybdate II and 0.4 ml. of aminonaphtholsulfonic acid reagent, followed by water to a volume of 10 ml. Establish the density of a standard phosphate solution as follows: Transfer 5 ml. of the stock phosphate standard, containing 0.4 mg. of P, to a 50-ml. volumetric flask, make up to volume with 10 per cent trichloroacetic acid, and mix. Transfer 5 ml. of this dilute standard, containing 0.04 mg. of phosphorus, to a suitable container, add 1 ml. of Molybdate II

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[218] Reagents Required: *10 Per Cent Trichloroacetic Acid.* Dissolve 10 g. of reagent-grade trichloroacetic acid in water and dilute to 100 ml.

10 N Sulfuric Acid. Carefully add 450 ml. of concentrated sulfuric acid to 1300 ml. of water. To check, dilute 10 ml. of this solution to 100 ml. in a volumetric flask, mix, and titrate a 10-ml. portion with standard 1 N sodium hydroxide. From the titration results, adjust the original solution if necessary to make it exactly 10 N.

Molybdate I. Dissolve 25 g. of reagent-grade ammonium molybdate in about 200 ml. of water. In a 1-liter volumetric flask place 500 ml. of 10 N sulfuric acid. Add the molybdate solution and dilute with washings to 1 liter with water. Mix. Stable indefinitely.

Molybdate II. Dissolve 25 g. of reagent-grade ammonium molybdate in about 200 ml. of water. In a 1-liter volumetric flask place 300 ml. of 10 N sulfuric acid. Add the molybdate solution and dilute with washings to 1 liter with water. Mix. Stable indefinitely.

Aminonaphtholsulfonic Acid Reagent. Place 195 ml. of 15 per cent sodium bisulfite solution (see below) in a glass-stoppered cylinder. Add 0.5 g. of 1,2,4-aminonaphtholsulfonic acid (satisfactory material can be obtained from Eastman Kodak Co., Rochester, N.Y.). Add 5 ml. of 20 per cent sodium sulfite (see below). Stopper and shake until the powder is dissolved. If solution is not complete, add more sodium sulfite, 1 ml. at a time, with shaking, but avoid an excess. Transfer the solution to a brown-glass bottle and store in the cold. This solution is usable for about four weeks, if kept as described.

15 Per Cent Sodium Bisulfite. To 30 g. of reagent-grade sodium bisulfite in a beaker add 200 ml. of water from a graduated cylinder. Stir to dissolve, and if turbid allow to stand well-stoppered for several days and filter. Keep well-stoppered.

20 Per Cent Sodium Sulfite. Dissolve 20 g. of reagent-grade anhydrous sodium sulfite in water, dilute to 100 ml., and filter if necessary. Keep well-stoppered.

Standard Phosphate Solution. Dissolve exactly 0.351 g. of pure dry monopotassium phosphate in water and transfer quantitatively to a 1-liter volumetric flask. Add 10 ml. of 10 N sulfuric acid, dilute to the mark with water, and mix. This solution contains 0.4 mg. of phosphorus in 5 ml. It is stable indefinitely.

and 0.4 ml. of aminonaphtholsulfonic acid reagent, dilute to 10 ml. with water, and mix. Allow to stand 5 minutes and determine the density in the photometer whose zero is set with a blank as described above.

CALCULATION. *For colorimetric measurement:* Since the 5 ml. of filtrate taken represent 1 ml. of original sample, and the standard containing 0.4 mg. of P is in ten times the volume of the unknown, the calculation becomes:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{0.4}{10} \times 100$$
$$= \text{mg. inorganic P per 100 ml. whole blood, plasma, or serum}$$

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.04 \times 100$$
$$= \text{mg. inorganic P per 100 ml. whole blood, plasma, or serum}$$

The density of the standard at 660 mμ in a 1-cm. cuvette is approximately 0.500 (see Fig. 166); at 720 mμ, the peak position of the curve, the density is about 10 per cent greater. Under these conditions the limit of accurate measurement corresponds to an inorganic phosphate content of approximately 7 to 8 mg. per cent, and this range is proportionately reduced with photometric measurement at greater depth of solution. To permit the covering of a greater range of phosphate concentration, the amount of sample taken for protein precipitation may be decreased, e.g., 1 ml. (or 0.5 ml.) of whole blood, serum, or plasma is treated with 10 per cent trichloroacetic acid at a final volume of 10 ml., and 5 ml. of filtrate taken for analysis as described. If this is done, the calculation is the same except that the final result is multiplied by 2 (or 4, if 0.5 ml. of sample was taken). The color obtained in this procedure shows little change between 5 and 20 minutes after adding the aminonaphtholsulfonic acid reagent, and the agreement with Beer's law is excellent, permitting calculation of results in terms of the density of a simultaneously prepared standard and eliminating the necessity for a calibration curve.

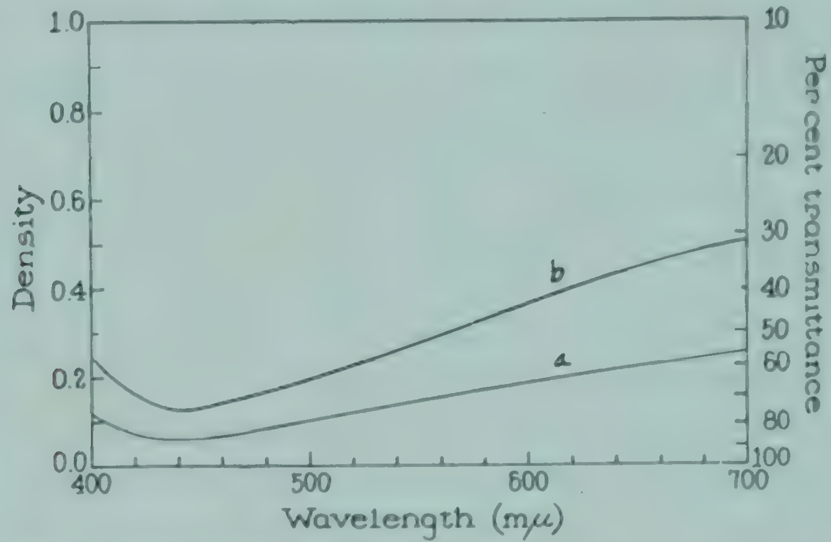

FIG. 166. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN FISKE-SUBBAROW PHOSPHATE METHOD.

For standards containing (*a*) 0.02 mg., and (*b*) 0.04 mg. phosphorus. Solution depth, 1 cm.

Interpretation. The normal inorganic phosphorus content of the blood plasma or serum is about 5 mg. per 100 ml. in infants and children and 3.7 mg. in the adult. In severe nephritis inorganic phosphorus may rise 15 to 20 mg. and may bear a relation to the acidosis found in such cases. Clinically, the most important changes are those in rickets. In the rickets of children or in experimental rickets produced in animals by low-phosphorus diets the inorganic phosphorus of the blood may fall to 2 mg. or lower. Treatment with antirachitic vitamin or ultraviolet radiation increases the phosphorus of the blood and leads to recalcification of the rachitic lesions. Occasionally rickets may be accompanied by high inorganic phosphorus of the blood, and the tendency of irradiated ergosterol to restore normal phosphorus values may be independent of its calcifying activity. During the healing period following fracture of a bone, an increase in plasma phosphates is sometimes observed. The phosphate level of the plasma of children rises during the summer and falls during the winter. Rickets has its greatest incidence during the late winter, reaching its peak in March. These findings may be correlated with the degree of exposure to solar ultraviolet rays. Injections of insulin decrease the phosphate of the plasma.

2. Determination of Total Acid-soluble Phosphorus: Principle. The organic matter in an aliquot of a trichloroacetic acid filtrate is destroyed by digestion with sulfuric acid and subsequent oxidation with 30 per cent hydrogen peroxide. The phosphate-containing solution is then analyzed for phosphate by the method of Fiske and SubbaRow.

Procedure.[219] **Prepare a trichloroacetic acid filtrate of whole blood, plasma, or serum as described for the determination of inorganic phosphate. Transfer 2 ml. of this filtrate to a test tube, 200 by 25 mm., and add 2.5 ml. of 5 N sulfuric acid and a quartz chip to minimize bumping. Place in a slanting position over a microburner, with the burner tip about 2 cm. below the bottom of the tube, or suspend in a wire basket about 1.5 inches above an electric hot plate. After evaporation is complete and the mixture turns brown or black with no further change, remove the tube, cool slightly, and add 1 drop of 30 per cent hydrogen peroxide, allowing the drop to fall directly into the digestion mixture. Replace the tube and continue heating. The contents of the tube should become colorless; if not, repeat the addition of hydrogen peroxide and heating. When colorless, cool the tube, add a few ml. of water, and heat to boiling momentarily. Cool again and transfer the contents of the tube to a 25-ml. volumetric flask, with washings until the flask is about half full. Add 2.5 ml. of 2.5 per cent ammonium molybdate solution, followed by 1 ml. of aminonaphtholsulfonic acid reagent. Dilute with water to the 25-ml. mark and mix. Allow to stand 5 minutes, then read in the colorimeter or photometer.**

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[219] Reagents Required: *10 Per Cent Trichloroacetic Acid Solution, Molybdate I Solution, Aminonaphtholsulfonic Acid Reagent,* and *Standard Phosphate Solution* as described for the determination of Inorganic Phosphate (p. 631), and in addition:

5 N Sulfuric Acid. Fill a 250-ml. volumetric flask to the mark with 10 N sulfuric acid solution (see p. 631). Pour the contents of the flask into a 500-ml. volumetric flask, with rinsings, and dilute to the mark with water. Mix. Stable indefinitely.

2.5 Per Cent Ammonium Molybdate Solution. Dissolve 2.5 g. of reagent-grade ammonium molybdate in water, transfer to a 100-ml. volumetric flask, fill to the mark, and mix. As soon as any appreciable amount of sediment forms in this solution, it should be discarded.

30 Per Cent Hydrogen Peroxide. Only the highest purity, essentially phosphate-free material may be used. The products of Merck and of J. T. Baker are known to be satisfactory. *This reagent is extremely corrosive to the skin* and must be handled carefully. Keep in the cold, and use a medicine dropper for dispensing.

For colorimetric measurement, compare against a standard prepared as described above for the determination of blood inorganic phosphate. For photometric measurement, the most accurate procedure is to run a digested blank and a digested standard along with each series of unknowns. This corrects for any phosphate or other factors in the reagents which may affect the final results. Digested blank: 2 ml. of 10 per cent trichloroacetic acid, treated with sulfuric acid, evaporated, oxidized with hydrogen peroxide, and color reagents added, exactly as described for the unknown. Digested standard: 1 ml. of standard phosphate solution, containing 0.08 mg. of phosphorus, plus 2 ml. of 10 per cent trichloroacetic acid, treated with sulfuric acid, evaporated, oxidized with hydrogen peroxide, and color developed, exactly as described for the unknown. The color of the digested blank and standard is developed at a final volume of 25 ml., as with the unknown. Set the photometer to zero density with the blank, and determine the densities of the standard and the unknown, using the same wavelength as specified for the determination of inorganic phosphate on p. 632.

CALCULATION. *For colorimetric measurement:* Since the standard contains 0.4 mg. of P in a final volume of colored solution four times that of the unknown, and the 2 ml. of unknown filtrate represent 0.4 ml. of original sample, the calculation is as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{0.4}{4} \times \frac{1}{0.4} \times 100 = \text{mg. total acid-soluble phosphorus per 100 ml. blood, plasma, or serum}$$

If the reagents, particularly the trichloroacetic acid and the hydrogen peroxide, contain significant amounts of phosphorus, the quantity present must be established by a blank digestion and analysis and suitable corrections made.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.08 \times \frac{1}{0.4} \times 100 = \text{mg. total acid-soluble phosphorus per 100 ml. blood, plasma, or serum}$$

The conditions for photometric measurement have been presented in connection with the determination of inorganic phosphate.

Interpretation. The acid-soluble phosphorus content of normal blood is approximately 25 to 30 mg. per 100 ml. of whole blood. The total acid-soluble phosphorus includes the inorganic phosphate as well as those organic phosphate esters which are not present as lipide or nucleoprotein material. Such esters include hexosephosphate, diphosphoglyceric acid,[220] certain free nucleotides, and other compounds which have not been characterized. In the plasma, the total acid-soluble phosphorus is represented largely by inorganic phosphate. In the cells, the reverse is true, ester phosphate being relatively much more abundant; thus interpretation of total acid-soluble phosphorus values for whole blood must include a consideration of the relative volume of cells to plasma. There is some evidence that the acid-soluble phosphorus of blood is of significance in acid-base balance in the body, since it is found to be considerably depleted during experimental acidosis.

***3. Determination of Lipide Phosphorus.* See p. 589.**

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[220] Greenwald: *J. Biol. Chem.*, **63**, 339 (1925).

4. *Determination of Total Phosphorus.* Dilute 1 ml. of blood, plasma, or serum to 10 ml. with 0.9 per cent sodium chloride solution and mix. Transfer 2 ml. of this diluted solution to a 200 × 25 mm. test tube, add 2.5 ml. of 5 N sulfuric acid and a quartz chip, and heat over a microburner or electric hot plate as described for the determination of acid-soluble phosphorus on p. 633. Foaming may be minimized by blowing clean compressed air into the digestion tube through a fine-tipped glass tube. When the water has been driven off and the contents of the tube turn black, continue with the digestion, oxidation, color development, and measurement as described for the determination of acid-soluble phosphorus. Calculation of results is the same except that since the 2 ml. of sample taken for analysis represent 0.2 ml. of original sample instead of 0.4 ml. as in the determination of acid-soluble phosphorus, results are multiplied by 2 to give the total phosphorus, in mg. per 100 ml.

Determination of Phosphorus in Tissues and Other Biological Material. The colorimetric method described above may be used for tissues and other biological material. Dry-ashing may sometimes be necessary. If little organic matter is present, ignition with a small amount of sodium carbonate will suffice. In other cases magnesium nitrate (1 ml. of 10 per cent) is satisfactory. For tissues, carbonate-nitrate fusion mixtures are perhaps best. Porcelain dishes may generally be used, but blanks should be run on them. Some silica will not interfere. If platinum is used, dissolve most of the ash with water before adding acid to remove the last of the material, since nitrous acid attacks platinum. Evaporate the total solution to dryness in a beaker or porcelain dish (covered with a watch glass as long as carbon dioxide is evolved). Dissolve the residue in water, make to volume, and determine as usual.

DETERMINATION OF SERUM PHOSPHATASE ACTIVITY

Introduction. Normal blood serum contains several enzymes or groups of enzymes which catalyze the liberation of inorganic phosphate from phosphate esters such as glycerophosphate, phenylphosphate, etc. The most active phosphatase, and the one longest recognized, has an optimum pH of approximately 9, and is now known as "alkaline" phosphatase to distinguish it from a second or "acid" phosphatase which is of limited activity in normal serum but whose activity is of significance in certain pathological conditions and which has an optimum pH of approximately 5. Neither type of enzyme has any significant activity at the optimum pH of the other, although both are active at the pH of normal blood. The enzymatic activity of serum or plasma with respect to either or both enzymes is established in terms of the rate of hydrolysis of suitable phosphate ester substrates buffered to the proper pH.

Various methods have been proposed for the determination of phosphatase activity of serum. For "alkaline" phosphatase, the method of Bodansky[221] has perhaps found the widest application. In this method, the phosphate liberated on incubation of serum with buffered glycerophosphate at pH 8.6 is used as an index of phosphatase activity, one Bodansky unit corresponding to the liberation of 1 mg. of inorganic phosphate per 100 ml. of serum during a one-hour period of incubation under these conditions. In the Bodansky method, the liberated phosphate is determined with stannous chloride as reducing agent, and various corrections for the influence of protein precipitant and substrate on the phosphate determination are necessary; these have been established by the author. Shinowara, Jones, and Reinhart[222] claim that these correction factors become unnecessary under the proper conditions and likewise feel that "alkaline" phosphatase activity should be determined at pH 9.3, the optimum pH for this enzyme. They have described procedures for the determination of inorganic phosphate, "alkaline" phosphatase, and "acid" phosphatase on as little as 0.06 ml. of serum. King and Armstrong[223] proposed the determination of "alkaline" phosphatase activity in terms of the phenol liberated on incubation with buffered phenylphosphate, and Gutman and Gutman[224] have applied this procedure to the determination of "acid" phosphatase. A disadvantage of the King and Armstrong procedure is that the serum inorganic phosphate content, which is usually of importance along with phosphatase activity, must be determined separately with an entirely different set of reagents. In the procedures described here, the incubation procedure of the Bodansky method is used for "alkaline" phosphatase determination, with modification to permit the use of the method of Fiske and SubbaRow (see p. 630) for the determination of phosphate liberated; for "acid" phosphatase, the conditions prescribed by Shinowara, Jones, and Reinhart are followed, likewise modified to permit the use of the Fiske and SubbaRow phosphate method.

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[221] Bodansky: *J. Biol. Chem.*, **99**, 197 (1932); **101**, 93 (1933). This method is described in the eleventh edition of this book.

1. *Determination of "Alkaline" Phosphatase.*[225]

Procedure. **Collect about 5 ml. of whole blood in a centrifuge tube, allow to clot at room temperature, remove clot, then centrifuge (twice if necessary). The separated serum may be kept for several hours in the refrigerator, or for several days if frozen.**

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[222] Shinowara, Jones, and Reinhart: *J. Biol. Chem.*, **142**, 921 (1942).

[223] King and Armstrong: *Can. Med. Assoc. J.*, **31**, 376 (1934)

[224] Gutman and Gutman: *J. Biol. Chem.*, **136**, 201 (1940).

[225] Reagents Required: *"Alkaline Phosphate" Substrate.* Into a 100-ml. volumetric flask introduce successively 3 ml. of petroleum ether (B.P. 20° to 40° C., J. T. Baker Analyzed Special), about 80 ml. of distilled water, 0.5 g. of sodium β-glycerophosphate (Eastman), 0.424 g. of sodium diethyl barbiturate (Merck), and water to volume (read at interface between petroleum ether and aqueous solution). Empty (out of doors) into a 100-ml. glass stoppered pyrex bottle containing an inch layer of petroleum ether. Keep in the refrigerator. When multiples of 100 ml. are prepared, it is advisable to distribute the substrate into small bottles.

30 Per Cent Trichloroacetic Acid. Dissolve 30 g. of reagent-grade trichloroacetic acid in water and dilute to 100 ml. Stable indefinitely. To prepare a 10 per cent solution from this, dilute 1 volume with 2 volumes of water. To prepare a 5 per cent solution, dilute 1 volume with 5 volumes of water.

Standard Phosphate Solution. Place 6.25 ml. of the standard phosphate solution used for the determination of inorganic phosphate (see p. 631), containing 0.5 mg. of phosphorus, in a 100-ml. volumetric flask. Add 16.7 ml. of 30 per cent trichloroacetic acid solution, dilute to 100 ml. with water, and mix. This solution contains 0.04 mg. of phosphorus in 8 ml., in 5 per cent trichloroacetic acid. It should be stable indefinitely if stored in the cold.

Molybdate II, Aminonaphtholsulfonic Acid Reagent. See p. 631.

(a) INCUBATED SAMPLE. **Measure 9 ml. of "alkaline phosphate" substrate into a glass-stoppered cylinder[226] and place in an incubator or water bath at 37° C. until the fluid reaches incubator temperature. Add 1 ml. of serum, mix, note the time, and incubate for exactly 1 hour. Remove, cool in ice water for several minutes, and add 2 ml. of 30 per cent trichloroacetic acid. Mix, let stand a few minutes, and filter through a low-ash filter paper.**

(b) CONTROL SAMPLE. **At or near the end of the incubation period, prepare a control sample as follows: Measure 9 ml. of substrate into a glass-stoppered cylinder and add 2 ml. of 30 per cent trichloroacetic acid. With mixing add 1 ml. of serum, stopper, shake, and filter as above.**

When both filtrates are ready, transfer 8 ml. of each to cylinders or test tubes graduated at 10 ml. In a third similar container place 8 ml. of standard phosphate solution, containing 0.04 mg. of phosphorus. For photometric measurement, a fourth or blank tube is necessary. This contains 8 ml. of 5 per cent trichloroacetic acid alone. When all the tubes are ready, add to each 1 ml. of Molybdate II reagent, and mix. Add 0.4 ml. of aminonaphtholsulfonic acid reagent to each, dilute immediately to 10 ml. with water, and mix. Allow to stand 5 minutes for color development.

CALCULATION. *For colorimetric measurement,* compare both control and incubated unknowns against the standard. Since the 8 ml. of filtrate represent two-thirds of the total sample, the calculation is as follows:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.04 \times \frac{3}{2} \times 100 = \text{mg. inorganic phosphate per 100 ml. serum (control or incubated)}$$

For photometric measurement, read the unknowns and standard in a photometer which is set to zero density with the blank, using the same conditions as for the determination of inorganic phosphate described on p. 631. The calculation is as follows:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.04 \times \frac{3}{2} \times 100 = \text{mg. inorganic phosphate per 100 ml. serum (control or incubated)}$$

The *phosphatase activity* is the difference between the inorganic phosphate content of the incubated and control samples, expressed in mg. of phosphorus per 100 ml. Thus, if the control result is 4.0 mg. per cent, and the incubated result is 8.5 mg. per cent, the phosphatase activity is 8.5 − 4.0 = 4.5 Bodansky units per 100 ml. serum.

In colorimetric measurement, with the standard set at 20 mm., the control tube usually reads approximately 30 mm., and a phosphatase activity up to about 8 units may be accurately determined. In photometric measurement in a 1-cm. cuvette, the limit is about 12 units. For activities greater than this, the phosphate analysis is repeated on a smaller portion of filtrate from the incubated serum, making up to 8 ml. volume with 5 per cent trichloroacetic acid, and correcting the calculations accordingly. If the liberated phosphate is 60 mg. per cent or higher, inhibition of hydrolysis occurs and the incubation is repeated with the time shortened to 30 or 15 minutes, in which case results are multiplied by 1.8 or 3.3 respectively. The result on the control sample is essentially an analysis for serum inorganic phosphate, but includes any effect of glycerophosphate on color development. This effect is frequently very low or negligible, in which case the results on the control serum may be accepted as the measure of the serum inorganic phosphate, and reported as such along with the phosphatase values.

Interpretation. The serum "alkaline" phosphatase activity in normal adults ranges from 1.5 to 4.0 units per 100 ml. (average 2.7) and in normal children 5 to 12 units (average 8.0). These values are greatly exceeded in polyostotic Paget's disease (up to 50 times), rickets (up to 20 times), hyperparathyroidism (10 times). Smaller increases are observed in a number of other diseases of osseous origin. High serum phosphatase is a manifestation of processes that cause rapid growth of bone in the normal young, of new bone (repair), and of calcified and uncalcified pathological bone. Among nonosseous conditions in which increased phosphatase activity is observed are acute catarrhal jaundice and other cases of liver involvement. Under certain conditions the serum phosphatase activity may be used in the differential diagnosis of liver disease.

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[226] Ordinary test tubes and clean rubber stoppers may be used.

2. *Determination of "Acid" Phosphatase.*[227] The procedure is exactly the same as for "alkaline" phosphatase, except that the buffered "acid phosphate" substrate of Shinowara, Jones, and Reinhart is used for the incubation and for the control sample. Calculation of results is the same as for "alkaline" phosphatase, the unit of "acid" phosphatase activity being defined as equivalent to the liberation of 1 mg. per cent of inorganic phosphate during 1 hour incubation at pH 5.0.

Interpretation. In terms of the unit above defined, normal serum contains from 0.0 to 1.1 units of acid phosphatase activity, with no significant elevation observed except in cases of carcinoma of the prostate with metastases, when values as high as 30 units or more have been observed. It should be pointed out here that the King and Armstrong unit as used by Gutman and Gutman[224] is defined in terms of the liberation of 1 mg. per cent of phenol, and is approximately double the value of the phosphate unit here described. Results by the method described here will therefore tend to be about half as high as those obtained by the use of phenylphosphate as substrate.

DETERMINATION OF CHOLINESTERASE ACTIVITY

Introduction. Enzymes which catalyze the hydrolysis of acetylcholine are known as cholinesterases and are found in both the red cells and the plasma of the blood, as well as in most other body tissues. They are particularly active in nervous tissue, as discussed on p. 297. Available evidence indicates that cholinesterase is not a single enzyme but rather a name for a group of enzymes which can be further characterized as *true cholinesterase* and *pseudocholinesterase* in terms of tissue location, physiological function, and substrate specificity. The red blood cell enzyme is considered to be a true cholinesterase, as is the brain enzyme, since it is found that these enzymes act more rapidly on acetylcholine than on other choline esters such as butyrylcholine and benzoylcholine. The plasma enzyme is a pseudocholinesterase in the sense that it will act rapidly not only on acetylcholine but also on a variety of other esters. Since the red cell and plasma enzymes differ in physiological function (see discussion below) as well as in substrate specificity, determination of whole-blood cholinesterase activity is relatively meaningless, the analysis being carried out on the separated red cells or plasma as required.

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[227] Reagents Required: "*Acid Phosphate*" *Substrate.* This is identical with the "alkaline phosphate" substrate already described except that sufficient acetic acid is incorporated to bring the pH to 5.0. Into a 100-ml. volumetric flask introduce successively 3 ml. of petroleum ether, about 80 ml. of water, 0.5 g. of sodium β-glycerophosphate, 0.424 g. of sodium diethylbarbiturate, and 5 ml. of 1 N acetic acid. Dissolve by mixing, and add water to bring the aqueous meniscus to the 100-ml. mark. Keep in the refrigerator. The pH of the final solution should be checked and, if it is not 5.0, adjusted to that value by the addition of dilute acid or alkali as necessary.

Other reagents required are those described for the determination of "alkaline" phosphatase, with the exception of the substrate.

Estimation of cholinesterase activity is based on measurement of the rate of hydrolysis of the substrate (usually acetylcholine) under specified conditions. When acetylcholine is hydrolyzed, acetic acid is formed, and measurement of rate of enzyme action is conveniently made in terms of rate of acid production. The standard method for measuring cholinesterase activity is that of Ammon,[228] based upon the use of the Warburg apparatus (see p. 334), for the manometric measurement of CO_2 liberated from bicarbonate by the enzymatic production of acid. A variety of simpler methods based upon acid production have been described, of which the method of Michel,[229] described here, is believed to be one of the most satisfactory. A colorimetric procedure based upon the determination of acetylcholine present before and after treatment with the enzyme has been described by Hestrin.[230] It is not always possible to compare cholinesterase activities obtained by various methods because of the sensitivity of the enzyme to temperature, pH, and particularly substrate concentration, and these conditions are rarely the same from one method to another.

Method of Michel:[229] ***Principle.*** The enzyme in an aliquot of diluted plasma or red cell hemolyzate is allowed to act on acetylcholine in a standard buffer solution for a measured time (usually 1–2 hours). The pH of the mixture is measured at the beginning and at the end of this time. The action of the enzyme on the substrate produces acid which lowers the pH of the mixture; the rate of change in pH is therefore a measure of enzymatic activity.

Procedure.[231] (a) SEPARATION OF RED CELLS AND PLASMA. **Place approximately 5 ml. of freshly drawn blood, heparinized to prevent clotting, in a graduated centrifuge tube and centrifuge at 2,000 r.p.m. for 15 minutes. Remove the**

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[228] Ammon: *Arch. f. d. ges. Physiol.*, **233**, 57 (1933).

[229] Michel: *J. Lab. Clin. Med.*, **34**, 1564 (1949).

[230] Hestrin: *J. Biol. Chem.*, **180**, 249 (1949).

[231] Reagents Required: *Buffer Solution I (for Red Cells).* Dissolve 4.12 g. sodium barbital, 0.54 g. KH_2PO_4, and 44.7 g. KCl in about 900 ml. of distilled water. Add 28.0 ml. 0.1 N hydrochloric acid solution with mixing, dilute to 1 liter, and mix. Check the pH, which should be 8.10 at 25° C.; if necessary, adjust to the proper pH by the careful further addition of 0.1 N acid or alkali as required. Add a few drops of toluene and keep in the refrigerator.

Buffer Solution II (for Plasma). Dissolve 1.24 g. sodium barbital, 0.17 g. KH_2PO_4 and 17.54 g. NaCl in about 900 ml. distilled water. Add 11.6 ml. 0.1 N hydrochloric acid with mixing, dilute to 1 liter, and mix. The pH should be 8.00 at 25° C.; if not, adjust as described above. Preserve with toluene and keep in the refrigerator.

Acetylcholine Substrate (0.11 M) for Red Cells. Dissolve 2.00 g. of high-grade acetylcholine chloride in 100 ml. of distilled water. Add toluene as preservative and keep in the refrigerator.

Acetylcholine Substrate (0.165 M) for Plasma. Dissolve 3.00 g. acetylcholine chloride in 100 ml. of distilled water. Preserve with toluene and store in the cold.

Saponin Solution. 0.01 per cent saponin in distilled water.

Both Buffer Solution I and Buffer Solution II show a slow decrease in pH and buffer capacity after standing for several weeks, and should be checked with a pH meter before use. If the pH_1 reading in a determination is more than 0.03 pH units below 8.00, a fresh supernatant plasma with a capillary pipet and set it aside if desired for analysis as described under (c) below. Add 2 to 3 volumes of 0.9 per cent NaCl solution to the red cells, mix well, and again centrifuge for 15 minutes. Discard the supernatant saline and repeat the washing procedure, this time centrifuging for 20 minutes. Note the volume of packed red cells, and remove the supernatant saline to a point where the volume of cells and saline is twice the volume of cells alone. Mix the cells well in the remaining saline, thus obtaining a 1:1 dilution of the washed cell suspension in saline. Transfer 0.4 ml. of this cell suspension to a test tube containing 9.6 ml. of 0.01 per cent saponin solution. This hemolyzed red cell solution, of which 1 ml. represents 0.02 ml. of red cells, is used for the procedure described under (b).

(b) MEASUREMENT OF RED CELL ACTIVITY. Transfer 1 ml. of hemolyzed red cell solution, prepared as described above, to a small beaker[232] containing 1 ml. of Buffer Solution I. Mix and place the beaker in a water bath at 25° C. for 10 minutes, for temperature equilibration. Measure the pH of the mixture, using a pH meter and reading to the nearest 0.01 unit. Return the beaker to the thermostat. Note the time, and add 0.2 ml. of 0.11 M acetylcholine solution with rapid mixing. Allow the beaker to stand in the thermostat for 1 to 1½ hours. Again measure the pH and note the time. Calculate results as described below.

(c) MEASUREMENT OF PLASMA ACTIVITY. Dilute 0.2 ml. of plasma to 10 ml. with water, and mix. Transfer 1 ml. of this diluted plasma to a small beaker containing 1 ml. of Buffer Solution II. Place in a thermostatically regulated water bath at 25° C. for 10 minutes; then measure the pH as described under (b) above. Note the time; then add 0.2 ml. of 0.165 M acetylcholine solution with rapid mixing. Carry out the subsequent steps as described under (b) above.

CALCULATION. The cholinesterase activity of the sample in units of ΔpH per hour is calculated as follows:

$$\Delta\text{pH/hr.} = \left(\frac{\text{pH}_1 - \text{pH}_2 - b}{t}\right) f$$

where pH_1 and pH_2 are the initial and final pH readings respectively, t is the time in hours between the mixing with acetylcholine and the time of reading pH_2, and b and f are correction factors obtained from the following table, b being a correction for non-enzymatic hydrolysis of substrate, and f correcting for the effect of pH change on enzyme activity relative to buffer capacity.

The results obtained by the procedure described here represent the cholinesterase activity for 0.02 ml. of red cells or plasma, expressed in units of ΔpH per hour. If the activity per ml. of red cells or plasma is desired, multiply the results by 50. To express results in terms of per cent activity relative to some normal or previous value on the same individual, multiply the ΔpH/hour value found by 100 and divide by the normal or previous ΔpH/hour value. For example, Michel found the mean normal ΔpH/hour value for red cell cholinesterase activity in 12 subjects to be 0.753. If an unknown sample gave a value of 0.550, then the activity in terms of *per cent of normal* would be 0.550/0.753 × 100, or 73 per cent.

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buffer solution should be prepared. Pure acetylcholine chloride solutions will produce a pH change of less than 0.01 pH units when added to Buffer I or Buffer II in the proportions specified in the text and in the absence of enzyme. Any greater change indicates decomposition of the solution, and a fresh solution should be prepared.

[232] Beakers which are supplied with most commercial pH meters for determining pH on small volumes (2-5 ml.) of solution are very satisfactory. It is necessary to shake the beaker containing the reaction mixture for a few seconds after immersing the electrodes in order to obtain rapid equilibrium.

CORRECTION FACTORS FOR CHOLINESTERASE DETERMINATION

pH_2	Red Cell Cholinesterase		Plasma Cholinesterase	
	b	*f*	*b*	*f*
7.9	0.03	0.94	0.09	0.98
7.8	0.02	0.95	0.07	1.00
7.7	0.01	0.96	0.06	1.01
7.6	0.00	0.97	0.05	1.02
7.5	0.00	0.98	0.04	1.02
7.4	0.00	0.99	0.03	1.01
7.3	0.00	1.00	0.02	1.01
7.2	0.00	1.00	0.02	1.00
7.1	0.00	1.00	0.02	1.00
7.0	0.00	1.00	0.01	1.00
6.8	0.00	0.99	0.01	1.00
6.6	0.00	0.97	0.01	1.01
6.4	0.00	0.97	0.01	1.02
6.2	0.00	0.97	0.01	1.04
6.0	0.00	0.99	0.01	1.09

Interpretation. In a series of 12 normal adult males, Michel found a mean value of 0.703 ΔpH per hour units for plasma and a value of 0.753 for red cells, by the method described here. Studies by a variety of methods on the red cell and plasma enzyme activities of normal adults have shown that there is no sharply defined level of cholinesterase activity which may be called "normal"; though the enzymatic activity of the blood of a particular individual does not vary significantly during health, wide variations exist between individuals in a normal group. The method is therefore more satisfactory when used for following changes in cholinesterase activity within a particular individual than for establishing whether enzymatic activity is normal or abnormal. The factors determining the maintenance of cholinesterase activity are not too clearly understood, particularly with regard to the red cell enzyme. The plasma enzyme appears to be similar to the other plasma proteins which originate in the liver, since it decreases during liver disease and returns to normal levels on recovery. Low plasma levels have also been reported in malnutrition[233] and pernicious anemia.[234] The most striking lowering of both plasma and red cell enzyme is found after treatment with the anticholinesterase drugs (DFP, physostigmine, neostigmine, etc.; see p. 298) and after exposure to certain organic insecticides such as Parathion and Schradan. Determination of blood cholinesterase levels is of significant diagnostic value in these conditions.

DETERMINATION OF SULFUR

Introduction. Of the total sulfur of whole blood, a portion is present as the inorganic sulfate ion, another portion is in the form of various nonprotein organic compounds which may be present (glutathione, ergothioneine, etc.), most of which are found chiefly in the red cells, and the remainder is represented by the sulfur-containing amino acids of the proteins present. In the analysis of serum or plasma, inorganic sulfate is ordinarily determined by isolation as the benzidine salt, followed by colorimetric[235] or titrimetric[236] estimation of the benzidine component. Nephelometric[237] and gasometric[238] methods for inorganic sulfate have also been described. Ethereal or conjugated sulfate (i.e., the increment in inorganic sulfate produced by acid hydrolysis of the protein-free sample) appears to be present, if at all, in such small amount in human plasma as to come within the limits of error in the two analyses necessary for its estimation. Significant values may be found for the plasma of species other than man. Total sulfur is determined by complete oxidation of organic matter, followed by estimation of inorganic sulfate present.

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[233] McCance: *Proc. Roy. Soc. Med.*, **43**, 272 (1950). See however Saunders *et al.*: *J. Nutrition*, **47**, 191 (1952).

[234] Cline, Johnson, and Johnson: *Southern Med. J.*, **41**, 374 (1948).

1. Determination of Inorganic Sulfate in Serum** (**Method of Letonoff and Reinhold**):*[239] ***Principle. Serum is deproteinized with uranium acetate. The sulfate in the filtrate is precipitated as benzidine sulfate, then washed, dissolved, and determined colorimetrically after treatment with sodium β-naphthoquinone-4-sulfonate.

Procedure. **Six ml. of 0.4 per cent uranium acetate solution are measured into a 15-ml. centrifuge tube, 2 ml. of nonhemolyzed serum are added slowly, and, after mixing by inverting four times, the mixture is centrifuged for 10 minutes. Four ml. of clear centrifugate are measured into another centrifuge tube (selected so that the tip will retain precipitates). One ml. of glacial acetic acid and 9 ml. of benzidine solution[240] are added. The tube is capped, placed in ice water for at least ½ hour, then centrifuged for 15 minutes at 3000 r.p.m. The supernatant fluid is decanted and discarded, and the tube permitted to drain in an inverted position for 3 minutes. Fourteen ml. of acetone are added. The precipitate is suspended in the acetone, then again centrifuged for 15 minutes at high speed. The acetone is decanted and the tube allowed to drain 5 minutes. After the mouth of the tube has been wiped, 1 ml. of a 1 per cent solution of sodium borate in 0.1 N sodium hydroxide is added and the precipitate dissolved by stirring. (The tube may be placed in warm water at 60° C. if solution is slow.) Finally, 10 ml. of water and 1 ml. of the color reagent[241] are added. The solutions are mixed and allowed to stand 5 minutes, then 2 ml. of acetone are added. At the same time, 2 standards are prepared by measuring 2 and 5 ml. of benzidine hydrochloride solu-**

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[235] For a colorimetric method different from the one described in the text, see Cuthbertson and Tompsett: *Biochem. J.*, **25**, 1237 (1931); Pirie: *Biochem. J.*, **28**, 1063 (1934).

[236] Power and Wakefield: *J. Biol. Chem.*, **123**, 665 (1938).

[237] Denis and Reed: *J. Biol. Chem.*, **71**, 191 (1926).

[238] Van Slyke, Hiller, and Berthelsen: *J. Biol. Chem.*, **74**, 659 (1927).

[239] Letonoff and Reinhold: *J. Biol. Chem.*, **114**, 147 (1936). This method is said to give lower results than other methods because of the absence of the hydrolytic effect of acid protein precipitants.

[240] One per cent benzidine in acetone, filtered and stored in a brown bottle in the refrigerator. It should be discarded when it becomes highly colored.

[241] *Color Reagent.* Dissolve 0.15 g. of pure sodium β-naphthoquinone-4-sulfonate in 100 ml. of distilled water. The solution will keep about 2 weeks in the cold. Each sample of this reagent should be tested by treating 2 ml. and 4 ml. of the working standard solution of benzidine hydrochloride with the color reagent, borate, water, and acetone as described below. Acceptable preparations do not deviate from the theoretical Beer's law relationship by more than 5 per cent.

tion[242] into 2 test tubes. One ml. of borate solution is added to each, followed by 8 ml. and 5 ml. of water respectively. One ml. of color reagent is added and the development of color carried out as described. The unknown solutions are compared with standards in the colorimeter.

CALCULATION. When *colorimetric* comparison is made with the 2-ml. benzidine standard:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.02 \times \frac{100}{1} = \text{mg. sulfate sulfur per 100 ml. serum}$$

For the stronger standard, replace 0.02 in the above equation by 0.05.

Precise *photometric* data for this color are not available. Since the reagent and reaction resemble that for the amino-acid determination (p. 565), satisfactory photometric measurement should be possible at 490 mμ, against a blank prepared by treating 10 ml. of water with 1 ml. of borate, 1 ml. of color reagent, and 2 ml. of acetone.

Interpretation. Normal human blood serum contains approximately 1 mg. of inorganic sulfate (as S) per 100 ml. In nephritis with retention, the inorganic sulfate values are increased. High values have also been reported in pyloric or intestinal obstruction, in leukemia, and in diabetes.

2. Determination of Total Sulfur in Biological Material (Method of Stockholm and Koch):[243] Principle. The complete oxidation of biological material for the sulfur determination is often a difficult procedure. The following method in which strong hydrogen peroxide and nitric acid are used is believed to be the most effective.

Procedure. **Into a 100-ml. nickel crucible (50 × 70 mm.) containing 10 ml. of 25 per cent sodium hydroxide solution, introduce 0.5 to 2.0 g. of the substance. The covered crucible is then heated on the steam bath until the mass is almost dry. This requires several hours, but causes considerable decomposition of the complex substances present, so that the sulfur in particular can later be easily oxidized. In case the evaporation has proceeded too rapidly, it is best to add again 10 ml. of water and to repeat the slow evaporation. To the slightly moist material 5 ml. of 30 per cent H_2O_2[244] are added very gradually. In some cases it is necessary to stir the mass with a glass rod or to add a few drops of water so as to distribute the reagent properly. During this treatment the heating is continued on the steam bath.**

The material thus partially oxidized is next transferred to a 300-ml. Kjeldahl flask, acidified with nitric acid, and concentrated over a free flame until salts begin to separate. This concentrated solution is then oxidized, while boiling,

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[242] *Standard Benzidine Hydrochloride Solution.* 0.1606 g. of benzidine hydrochloride, purified as described below, is transferred to a 200-ml. volumetric flask, dissolved in water previously warmed to about 50° C., cooled, and diluted to volume. The solution should be stored in the cold. Ten ml. are equivalent to 1.0 mg. of sulfur. For a working standard, 10 ml. of this solution are diluted to 100 ml. with water. One ml. contains benzidine equivalent to 0.01 mg. of sulfur. The solution should be stored in the cold.

The benzidine hydrochloride is purified as follows: 5 g. of benzidine hydrochloride are dissolved in 200 ml. of 5 per cent hydrochloric acid by warming to about 50° C. Any insoluble residue is filtered. Twenty ml. of concentrated hydrochloric acid are added with continuous stirring. The solution is cooled in ice water for about 30 minutes, when the crystals that have formed are collected on a Buchner funnel. The material is washed with cold diluted hydrochloric acid (15 ml. of concentrated acid to 100 ml. of water). After removing the hydrochloric acid by suction, the crystals are washed with two 25-ml. portions of cold ethyl alcohol and four portions of ether. After all traces of ether are removed, the dry crystals are transferred to a brown bottle.

[243] Stockholm and Koch: *J. Am. Chem. Soc.*, 45, 1953 (1923).

[244] Merck's Superoxol.

by the gradual addition of fuming nitric acid and bromine until 10 ml. of acid and 40 to 50 drops of bromine have been used. With material low in, or free from, fat this treatment is usually sufficient to bring about complete oxidation of the organic matter. The solution is next evaporated almost to dryness and, after water has again been added, evaporation is repeated to remove most of the nitric acid. When the water solution of this material is not absolutely clear it is filtered and after it has been neutralized with sodium hydroxide and diluted to about 600 ml. it is acidified by the addition of 10 ml. of concentrated hydrochloric acid. Ten ml. of 0.1 N sulfuric acid are added, the mixture heated to boiling and 10 ml. of a 10 per cent barium chloride solution added, drop by drop. The boiling is continued for 10 to 15 minutes and then the mixture is heated for 10 to 12 hours before it is filtered and washed as usual. The filter paper and precipitate are cautiously burned and weighed. Blank estimations are made in exactly the same way thus correcting for the sulfuric acid added to facilitate precipitation.

DETERMINATION OF CALCIUM

Introduction. The calcium of blood is found entirely in the plasma, red cells apparently being devoid of calcium. Since most of the common anticoagulants for blood act by reaction with calcium, serum from clotted blood is ordinarily used for analysis; heparinized plasma may also be used Of the total serum calcium, about half appears to be combined with protein in some way, and little is known of its physiological significance. The remainder is dialyzable, and, according to McLean and Hastings, largely in the ionic form. The symptoms of hypo- and hypercalcemia appear to be associated chiefly with variations in this diffusible fraction. Serum calcium is usually determined by precipitation as an insoluble calcium salt, such as oxalate or phosphate, for which specific analytical methods are available.

1. Clark-Collip Modification of the Kramer-Tisdall Method:[245] ***Principle.*** Calcium is precipitated directly from the serum as oxalate and the latter is titrated with potassium permanganate. Sendroy[246] has shown that direct precipitation as oxalate from diluted serum gives accurate results, and that preliminary removal of protein is not necessary.

Procedure. Introduce into a graduated 15-ml. centrifuge tube 2 ml. of clear serum, 2 ml. of distilled water, and 1 ml. of 4 per cent ammonium oxalate solution. Mix thoroughly. Mixing is aided by holding the tube at the mouth and giving it a circular motion by tapping the lower end. The centrifuge tube should have an outside diameter of 6 to 7 mm. at the 0.1 ml. mark. Let stand for 30 minutes or longer.[247] Again mix the contents. Centrifuge for about 5 minutes at 1500 revolutions per minute. Carefully pour off the supernatant liquid and while the tube is still inverted let it drain in a rack for 5 minutes, resting the mouth of the tube on a pad of filter paper.[248] Wipe the mouth of the tube dry with a soft cloth. Stir up the precipitate and wash the sides of the tube with 3 ml. of dilute ammonia (2 ml. of concentrated am-

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[245] Clark and Collip: *J. Biol. Chem.*, **63**, 461 (1925); Tisdall: *J. Biol. Chem.*, **56**, 439 (1923); Kramer and Tisdall: *J. Biol. Chem.*, **47**, 475 (1921); Clark: *J. Biol. Chem.*, **49**, 487 (1921).

[246] Sendroy: *J. Biol. Chem.*, **152**, 539 (1944).

[247] Overnight standing is preferred, otherwise precipitation is sometimes incomplete.

[248] To insure uniform drainage the tubes should always be cleaned thoroughly by heating at approximately 100° C. for a few minutes in a cleaning mixture made by adding 1500 ml. of concentrated sulfuric acid to a solution of 200 g. of sodium dichromate in 100 ml. of water.

monia to 98 ml. of water)[249] directed in a very fine stream, from a wash bottle. Centrifuge the suspension and drain again as before. Add 2 ml. of approximately normal sulfuric acid (28 ml. of concentrated acid to a liter) by blowing it from a pipet directly upon the precipitate so as to break up the mat and facilitate solution. Place the tube in a boiling water bath for about 1 minute. Titrate with 0.01 normal potassium permanganate[250] to a definite pink color which persists for at least 1 minute. If necessary during the course of the titration warm the tube by placing in a water bath kept at 70° to 75° C. A microburet graduated in 0.02 ml. should be used.

CALCULATION. One ml. of 0.01 N $KMnO_4$ is equivalent to 0.2 mg. of Ca.

$$(x - b) \times 0.2 \times \frac{100}{2} = \text{mg. calcium per 100 ml. serum}$$

½ - 500
1 - 250
3·5 12·5

where x equals the number of ml. of permanganate required in the titration, and b is the blank, i.e., the number of ml. of permanganate required to titrate 2 ml. of the sulfuric acid solution to the usual end point.

Interpretation. Normal human blood serum contains from 9 to 11.5 mg. of calcium per 100 ml. corresponding to 4.5–5.7 milliequivalents per liter. Values for children are slightly higher than for adults. A slight decrease may be observed during the late months of pregnancy. After parathyroidectomy the blood calcium falls to a low level and the injection of parathyroid extract results in a considerable increase. In infantile tetany values of 3.5 to 7.0 mg. have been observed. In severe nephritis the calcium content may decrease to 7.0 mg. or less. There is apparently a reasonably close relationship between serum calcium, phosphate, and protein. Except in children and in cases where the calcium metabolism *per se* is abnormal, this relationship may be expressed by the following equation:[251]

$$\text{Ca (in mg. per cent)} = 7 - 0.255\ \text{P (in mg. per cent)} + 0.566\ \text{protein (in grams per cent)}$$

Thus interpretation of pathological variations in serum calcium should be based upon knowledge of the serum phosphate and protein values, unless these are known to be normal.

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[249] Wang (*J. Biol. Chem.*, **111**, 443 (1935)) recommends as the washing solution, 2 per cent ammonia in equal parts of alcohol, ether, and water, to avoid flotation of the precipitate. Stanford and Wheatley (*Biochem. J.*, **19**, 710 (1925)) wash twice with a thoroughly filtered, saturated aqueous solution of calcium oxalate, 15 ml. of which, upon acidifying and titrating with 0.01 N $KMnO_4$, should require not more than 0.25 ml.

[250] *0.01 N* $KMnO_4$. See Halverson and Bergeim: *J. Ind. Eng. Chem.*, **10**, 119 (1918). Dissolve 0.4 g. of pure potassium permanganate in 1 liter of redistilled water in a thoroughly clean Florence flask. Insert a funnel covered with a watch glass as a condenser and digest for several hours at near the boiling point. Cool, let stand overnight, and filter with gentle suction through a 3-inch Buchner funnel lined with ignited asbestos. Transfer to a perfectly clean glass stoppered bottle and keep in a dark place. (Permanganate solution prepared by dilution of 0.1 N solution must be standardized immediately before use as it deteriorates rapidly and comes to a constant value only after several days.) The 0.01 N permanganate is standardized against 0.01 N sodium oxalate which should keep for several months. Dry highest purity sodium oxalate in an oven at 100° to 105° C. for 12 hours. Dissolve exactly 0.67 g. of the oxalate in redistilled water, add 5 ml. of concentrated H_2SO_4, and dilute to 1 liter. Mix well. Transfer exactly 25 ml. of this solution to a 100-ml. Erlenmeyer flask, add 1 ml. of concentrated H_2SO_4, warm to about 70° C., and titrate with the $KMnO_4$ solution. The permanganate solution should be frequently restandardized although after the first few days, if carefully prepared and kept, it should not deteriorate more than 0.1 per cent per week.

[251] Peters and Eiserson: *J. Biol. Chem.*, **84**, 155 (1929); Oberst and Plass: *J. Biol. Chem.*, **92**, xiii (1931). See, however, Stearns and Knowlton: *J. Biol. Chem.*, **92**, xii (1931).

2. ***Method of Roe and Kahn:***[252] ***Principle.*** Calcium is precipitated from the protein-free serum filtrate as tricalcium phosphate, which is then determined colorimetrically by a procedure similar to that used for the determination of blood inorganic phosphate (p. 630). Kuttner and Cohen have described a micromodification using this principle, in connection with their method for phosphorus determination.

Procedure.[253] To 8 ml. of 10 per cent trichloroacetic acid in a small flask, add 2 ml. of serum. Mix well by shaking. Filter through a Ca-free filter paper (Whatman No. 42 is satisfactory). Transfer 5 ml. of filtrate to a 15-ml. conical graduated centrifuge tube, add 1 ml. of 25 per cent Ca-free sodium hydroxide solution, mix by tapping, and let stand 5 minutes. Add 1 ml. of 5 per cent trisodium phosphate solution, mix thoroughly by tapping, and set aside for 1 hour. Centrifuge for 2 minutes. Decant supernatant liquid, place tube in an inverted position in a small beaker containing a mat of clean gauze or filter paper, and allow to drain 2 minutes. Wipe mouth of tube dry with a clean cloth. Add from a bulb pipet with a fine tip 5 ml. of alkaline-alcoholic wash reagent, forcing the wash fluid in at first so as to break up the mat of tricalcium phosphate, and then washing down the sides of the tube. If necessary, use a stirring rod to break up the precipitate, rinsing down the rod with a little of the wash fluid. Centrifuge 2 minutes, decant, drain, and wipe the mouth of the tube as before. Repeat the washing procedure,[254] centrifuging, etc., with a second 5-ml. portion of wash reagent, centrifuging, decanting, draining, and wiping off excess fluid as above. To the residue in the centrifuge tube add 1 ml. of acid molybdate reagent and tap against the palm of the hand to effect complete disintegration and solution of the precipitate. When dissolved, add 10 ml. of water, mix by tapping, and set aside. Prepare a standard by transferring 10 ml. of the standard phosphate solution, containing phosphate equivalent to 0.1 mg. of calcium, to a second similar graduated tube, add 1 ml. of the acid molybdate reagent, and mix. For photometric measurement, prepare a blank tube containing 10 ml. of water and 1 ml. of acid molybdate. When all the tubes are ready, add to each 0.5

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[252] Roe and Kahn: *J. Biol. Chem.*, 81, 1 (1929). Kuttner and Cohen: *J. Biol. Chem.*, 75, 517 (1927). The latter procedure as applied to the Roe and Kahn method is described in the eleventh edition of this book.

[253] Reagents Required: The trichloroacetic acid, sodium hydroxide, and trisodium phosphate ($Na_3PO_4 \cdot 12\ H_2O$) solutions are prepared on the indicated basis from reagent-grade chemicals. The two alkaline solutions should be decanted occasionally from any sediment which forms and which might lead to errors if included in the analysis.

Alkaline-Alcoholic Wash Reagent. Mix 58 ml. of ethyl alcohol with 10 ml. of amyl alcohol and dilute to 100 ml. with water. Add 2 drops of 1 per cent phenolphthalein and 5 per cent Ca-free NaOH a drop at a time to a distinct pink (usually 2 to 3 drops).

Acid Molybdate Reagent. Dissolve 12.5 g. of reagent-grade ammonium molybdate in 400 ml. of water in a 500-ml. volumetric flask. Add slowly with shaking 100 ml. of concentrated sulfuric acid. Stable indefinitely.

Aminonaphtholsulfonic Acid Reagent. The same as described on p. 631 for the determination of inorganic phosphate.

Standard Phosphate Solution. Dissolve 2.265 g. of pure dry monopotassium phosphate in water, dilute to 1 liter in a volumetric flask, and mix. Add a little chloroform as a preservative. This stock solution contains 0.517 mg. of P per ml., equivalent to 1 mg. of Ca as calcium phosphate. The working standard is prepared by diluting 1 ml. of stock solution to 100 ml. with water. This standard should be prepared fresh daily. 10 ml. contain phosphate equivalent to 0.1 mg. of Ca.

[254] Roe and Kahn state that only one washing is necessary. Two washings provide a greater margin of safety.

ml. of aminonaphtholsulfonic acid reagent, followed by water to the 15-ml. mark, and mix immediately by inversion. Allow to stand 10 minutes before reading in the colorimeter or photometer. For photometric measurement, set the photometer to zero density at 660 mμ with the blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.1 \times \frac{100}{1} = \text{mg. calcium per 100 ml. serum}$$

The standard corresponds to 10 mg. per cent serum calcium, and will be satisfactory for practically all values encountered in analyses. For very low values (below 5 mg. per cent) dilute the unknown after color development to 12 ml. instead of 15 ml., and correct the calculations accordingly.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.1 \times \frac{100}{1} = \text{mg. calcium per 100 ml. serum}$$

The characteristics of the color and the conditions of photometric measurement are the same as those described for the determination of inorganic phosphate (p. 632). At 660 mμ, in a 1-cm. cuvette, the standard has a density of approximately 0.400, and all values of serum calcium may be accurately determined under these conditions.

The photometric blank will correct for any phosphate present in the color-producing reagents, but not for contamination by calcium in the earlier stages of the procedure. Since it is very difficult to obtain absolutely Ca-free reagents or filter paper, for precise work a correction for calcium already present should be made. This is best done by running a standard calcium solution[255] through the entire procedure (treatment with trichloroacetic acid, filtering, etc.) and comparing the value obtained with that expected. Thus if a standard equivalent to 10 mg. per cent calcium gives on analysis a value of 10.5 mg. per cent, the correction to be subtracted from serum results is 0.5 mg. per cent. It is sometimes suggested that using such a calcium standard, instead of a phosphate standard as described, will include the calcium present in the reagents and hence serve as a correction. This is only true if the unknown and standard should happen to contain exactly the same amounts of calcium; otherwise it is in error, since the constant calcium correction becomes a variable and unknown fraction of the total calcium present in the unknown.

Interpretation. See previous method.

Other Methods. Numerous other methods have been proposed for the determination of serum calcium, some of which require only very small quantities of serum. Instead of titrating the oxalate directly, Fiske and Adams[256] redissolve the washed oxalate in nitric acid, dry, add oxalic acid, dry again, ignite, and titrate the residue as calcium oxide. Kuttner and Cohen (*loc. cit.*), in a method applicable to 0.1 ml. of serum, ash the material, precipitate the calcium as phosphate, and analyze for phosphate colorimetrically. Gasometric methods include measurement of the carbon dioxide evolved in the reaction between oxalate and permanganate[257] or ceric sulfate,[258] or in the combustion of calcium picrolonate;[259] the latter procedure is applicable to as little as 0.02 ml. of serum. Sendroy[260] has described photometric methods likewise applicable to 0.02 ml. of serum.

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[255] Suspend 0.250 g. of pure dry calcium carbonate in a little water in a 1-liter volumetric flask, add dilute (1:10) HCl to dissolve, add a few ml. of acid in excess, dilute to 1 liter with water, and mix. This solution is stable indefinitely and contains 0.1 mg. of Ca per ml. It corresponds therefore to a serum with a Ca content of 10 mg. per cent.

[256] Fiske and Adams: *J. Am. Chem. Soc.*, **53**, 2498 (1931).

[257] Van Slyke and Sendroy: *J. Biol. Chem.*, **84**, 217 (1929).

[258] Sendroy: *J. Biol. Chem.*, **152**, 557 (1944).

[259] Van Slyke and Kreysa: *J. Biol. Chem.*, **142**, 765 (1942).

Determination of Calcium in Tissues and Other Biological Materials. Where the amount of organic matter to be destroyed is small, the material may be ashed in silica or platinum crucibles with the aid of a few drops of nitric acid to destroy the last carbon. Feces, milk, and bone may usually be handled in this way. Tissues low in calcium present a more difficult problem and Corley and Denis have suggested autoclaving such materials with alkali.[261] By using a microcalcium determination the amount of organic matter necessary to be destroyed is greatly decreased.

DETERMINATION OF MAGNESIUM

Introduction. Magnesium is found constantly in small amount in blood, being distributed about equally between cells and plasma. Plasma or serum is ordinarily used for analysis, usually after the calcium has been precipitated to prevent interference in the analysis. Most methods proposed depend upon the precipitation of magnesium as phosphate, followed by colorimetric phosphate analysis. Precipitation of magnesium with 8-hydroxyquinoline has also been used.[262] Hirschfelder and Serles[263] have described a colorimetric method based upon the color formed in alkaline solution in the presence of certain dyes. The clinical significance of blood magnesium is relatively unknown; in tissues (such as muscle) the magnesium ion has been shown to play an important part in certain enzyme systems associated with intermediary metabolism (see Chapter 33).

Method of Denis[264] Modified: Principle. After removal of calcium as oxalate the magnesium is precipitated as magnesium ammonium phosphate and the latter is estimated by a colorimetric phosphate determination. In the method here described, the Fiske-SubbaRow phosphate method is used. For a procedure based on Youngburg's adaptation of Kuttner's colorimetric phosphate method, see the eleventh edition of this book.

Procedure. **Precipitate the calcium from 2 ml. of serum as in the procedure for calcium in serum (see p. 644). After centrifuging, pipet 3 ml. of the supernatant fluid into a 15-ml. graduated centrifuge tube and add with stirring 0.5 ml. of a 5 per cent solution of ammonium phosphate containing 5 ml. of concentrated NH_4OH per liter, followed by 2 drops of concentrated NH_4OH. Let stand overnight. Centrifuge. Siphon off the supernatant fluid and wash the tube with 5 ml. of a mixture of 1 part of concentrated NH_4OH (sp. gr. 0.9) and 2 parts of water. Centrifuge and siphon off wash liquid. Repeat the washing a second and third time and then wash finally with 5 ml. of 75 per cent alcohol containing 10 ml. of concentrated NH_4OH per liter. Siphon off again and let stand in a warm place until the ammonia has evaporated.**

To the residue in the centrifuge tube add 1 ml. of the Molybdate I reagent used in the Fiske-SubbaRow phosphate method (p. 639), and tap to dis-

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[260] Sendroy: *J. Biol. Chem.*, **144**, 243 (1942). See also Sendroy: *J. Biol. Chem.*, **152**, 539 (1944). This latter paper contains an extensive bibliography of serum Ca methods.

[261] Corley and Denis: *J. Biol. Chem.*, **66**, 601 (1925).

[262] Glomaud: *Ann. chim. anal.*, **24**, 166 (1942).

[263] Hirschfelder and Serles: *J. Biol. Chem.*, **104**, 635 (1934).

[264] Denis: *J. Biol. Chem.*, **52**, 411 (1922). See also Briggs: *J. Biol. Chem.*, **52**, 349 (1922)

solve. When dissolved, add 5 ml. of water and set aside. Prepare a standard by placing 1 ml. of the Molybdate I reagent in a second graduated tube and adding 3 ml. of the standard phosphate solution (equivalent to 0.03 mg. of magnesium)[265] plus 2 ml. of water. For photometric measurement prepare a blank by placing 1 ml. of Molybdate I reagent plus 5 ml. of water in a third graduated tube. When all the tubes are ready, add to each 0.4 ml. of aminonaphtholsulfonic acid reagent, followed immediately by water to the 10-ml. mark. Mix and allow to stand 5 minutes before reading in the colorimeter or photometer. For photometric measurement, set the photometer to zero density at 660 mμ with the blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.03 \times \frac{100}{1.2} = \text{mg. magnesium per 100 ml. serum}$$

The value 1.2 represents the fact that 3 ml. of supernatant obtained from 2 ml. of serum, after precipitating the Ca as oxalate, correspond to 1.2 ml. of the original serum.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.03 \times \frac{100}{1.2} = \text{mg. magnesium per 100 ml. serum}$$

The characteristics of the color and the conditions of photometric measurement are the same as for the determination of inorganic phosphate (p. 630). At 660 mμ, the standard has a density of approximately 0.500 in a 1-cm. cuvette. Since this standard corresponds to a serum Mg content of 2.5 mg. per cent, up to 5 mg. per cent may be accurately determined under these conditions. For higher values, or for photometric measurement at a greater depth of solution, add 2 ml. of molybdate reagent to unknown, blank, and standard instead of the 1 ml. specified (use the same 3-ml. portion of standard) followed by water in each case to about 12 ml., then add 0.8 ml. of aminonaphtholsulfonic acid reagent and water to a 20-ml. final volume. There is no change in the calculations. Color development at a greater dilution is recommended rather than the analysis of a smaller portion of serum because it is not known whether or not precipitation of magnesium in this procedure will be quantitative at conditions other than those specified.

Interpretation. Normally about 1 to 3 mg. of Mg are found in 100 ml. of serum and about 1.6 mg. per 100 ml. of blood. No characteristic changes have been observed in pathological conditions.

DETERMINATION OF SODIUM

Introduction. The sodium of whole blood appears to be present entirely in the form of the sodium ion, with over 90 per cent of the total blood sodium present in the plasma. Plasma or serum is therefore ordinarily used for analysis, and for precise work precautions should be taken against the transfer of water and electrolytes between cells and plasma brought about by changes in the blood gas content. Sodium is commonly determined by precipitation in the form of uranyl zinc sodium acetate, which, while soluble in water, is relatively insoluble in alcohol or acetone.

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[265] Dissolve 0.560 g. of pure dry monopotassium phosphate in water to make 1000 ml. Add a few drops of chloroform to prevent growth of molds. Dilute 10 ml. of this stock solution to 100 ml. 1 ml. = 0.01 mg. of Mg. For other solutions, see the phosphate method (p. 630).

Prior to isolation of the sodium as the triple salt indicated, the proteins are precipitated[266] or the sample is ashed;[267] electrodialysis may also be used for separation of the sodium from interfering material.[268] The triple salt may be determined by gravimetric,[267] titrimetric,[266] or colorimetric[269] methods. Another reagent which has been proposed for the determination of sodium is pyroantimonate.[270] In the analysis of serum sodium, precision is essential because of the relatively small variations which are of clinical significance. The determination of sodium in tissues is similar to the procedures used for blood.[271]

1. Determination by Flame Photometry: Principle. The sample in solution is introduced in the form of a fine continuous spray into a nonluminous gas flame. The various ions present produce their characteristic flame spectra. By the use of proper color filters or other means, the emitted light characteristic of the ion being determined is isolated and allowed to excite a photoelectric cell. The response of the photoelectric cell is measured on a suitable meter. Under controlled conditions, the meter reading is a measure of the concentration of ion being determined. A calibration curve is established by analyzing a series of standard solutions of the ion being determined. From the meter reading of the sample being analyzed, the concentration is established by reference to the calibration curve.

Procedure. **The procedure varies with different types of instrument. Ordinarily a dilution of 1:250 is used for sodium analyses, i.e., 1 ml. of plasma or serum is diluted to 250 ml. with water. If an internal standard, e.g., a lithium salt, is required for the successful operation of the photometer, the required aliquot of a stock solution of such a substance is added to the volumetric flask before diluting to the mark. The solution is then analyzed according to the directions which accompany the instrument. In order to get the sodium content of the sample, the reading obtained is referred to a calibration curve previously obtained by the same procedure on a series of solutions of known sodium content.**

STANDARDS. Standard solutions covering the range from 100 to 150 milliequivalents of sodium per liter, at a dilution of 1:250, are usually adequate for calibration purposes. Prepare a stock standard containing 100 milliequivalents of sodium per liter by dissolving 5.85 g. of NaCl in water and diluting to 1 liter. Working standards are prepared from this stock standard by measuring out 4.0, 5.0 and 6.0 ml. aliquots into 1-liter volumetric flasks, and diluting to the mark with water. These standards represent respectively 100, 125, and 150 milliequivalents of sodium per liter at a 1:250 dilution. If the analytical procedure requires an internal standard such as lithium sulfate solution, add to each working standard 4 times the volume of lithium sulfate solution routinely added to the diluted plasma or serum, before diluting to the mark and mixing. To prepare a calibration curve, read each of these standards in the photometer, plot readings against equivalent sodium content in milliequivalents per liter, and draw a smooth curve to include the points.

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[266] Weinbach: *J. Biol. Chem.*, **110**, 95 (1935); Ball and Sadusk: *ibid.*, **113**, 661 (1936); Dregus: *Biochem. Z.*, **303**, 69 (1939).

[267] Butler and Tuthill: *J. Biol. Chem.*, **93**, 171 (1931); Barber and Kolthoff: *J. Am. Chem. Soc.*, **50**, 1625 (1928); **51**, 3233 (1929). An improved version of the Butler and Tuthill method has been described by Pisha and Speier: *Arch. Bioch. and Biophys.*, **37**, 258 (1952).

[268] Keys: *J. Biol. Chem.*, **114**, 449 (1936); Consolazio and Talbott: *ibid.*, **132**, 753 (1940); Sobel, Kraus, and Kramer: *ibid.*, **140**, 501 (1941).

[269] McCance and Shipp: *Biochem. J.*, **25**, 449 (1931); Salit: *J. Biol. Chem.*, **96**, 659 (1932); Hoffman and Osgood: *ibid.*, **124**, 347 (1938). This latter is a photometric procedure.

[270] Kramer and Tisdall: *J. Biol. Chem.*, **46**, 467 (1921); Kramer and Gittleman: *ibid.*, **62**, 353 (1924).

[271] For a micro-procedure applicable to small amounts of tissues and blood, see Clark, Levitan, Gleason, and Greenberg: *J. Biol. Chem.*, **145**, 85 (1942).

Standards should always be run both before and after analyzing a series of unknown solutions, to be sure that the calibration is constant; if there is considerable fluctuation, each unknown solution should be followed by a standard closely equivalent to it.

Routine precautions in the use of flame photometers include the use of gas and air for the flame which are as free from contaminating ions as possible; maintenance of a constant gas and air pressure; installation of the photometer in a room free from airborne contamination, particularly tobacco smoke; and continual checking of the zero setting and standard readings. Cleaning of the atomizer and flame chimney may also be necessary from time to time.

Interpretation. See under 2, below.

2. Method of Weinbach:[266] ***Principle.*** After deproteinization, the sodium in the filtrate is precipitated in alcoholic medium as the triple salt, uranyl zinc sodium acetate, which is washed, dissolved in water, and titrated with standard sodium hydroxide. It is claimed that phosphates present do not interfere. For a similar method in which phosphates are first removed, see Dregus.[266]

Procedure.[272] **To 1 volume of whole blood or cells (or 2 volumes of serum or plasma) in a small flask, add 7 (or 6) volumes of water, shake, and let stand until hemolysis is complete. Add rapidly, with shaking, 2 volumes of 20 per cent trichloroacetic acid, making a total of 10 volumes. (This deproteinization may be done with as little as 0.1 to 0.2 ml. of material.) Mix, let stand 10 minutes, and filter through ashless filter paper (or centrifuge if the sample is small). Transfer 1 ml. of the whole blood filtrate (or 0.5 ml. of the serum filtrate) to a 15-ml. centrifuge tube and add 5 ml. of the uranyl zinc acetate reagent. From a 1-ml. graduated pipet add 0.3 ml. of 95 per cent alcohol and let stand for 5 minutes. Again add 0.3 ml. of alcohol and let stand for a few minutes. This procedure is repeated, without greatly disturbing the precipitate, until 2.1 ml. of alcohol have been added, the entire process of precipitation taking about ½ hour. Centrifuge, decant, drain by inversion on a pad of filter paper, and wipe the mouth of the tube with a cloth. Wash the precipitate once by blowing in 10 ml. of acetone wash reagent; centrifuge, decant, drain on filter paper, and wipe the mouth of the tube. The precipitate, which is readily soluble in water, is then transferred quantitatively to a 100-ml. Erlenmeyer flask by blowing in three or four 5-ml. portions of water, which has been recently boiled and cooled, to drive off dissolved carbon diox-**

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[272] Reagents Required: *Uranyl Zinc Acetate Reagent.* Solution A: 77 g. uranyl acetate, $UO_2(CH_3COO)_2 \cdot 2H_2O$, and 14 ml. of glacial acetic acid are dissolved in about 400 ml. of water by stirring and heating on a steam bath, and diluted to 500 ml. in a volumetric flask. Solution B: 231 g. of zinc acetate ($3H_2O$) and 7 ml. of glacial acetic acid are likewise dissolved and made up to 500 ml. The two solutions are mixed while hot, allowed to stand at least 24 hours, and filtered.

Acetone Wash Reagent. A small amount of the triple salt, uranyl zinc sodium acetate, is prepared by adding 15 ml. of the uranyl zinc acetate reagent to 1 ml. of approximately 5 per cent NaCl with subsequent addition of about 5 ml. of 95 per cent alcohol in small portions. Filter with suction and wash the precipitate with 4 or 5 small portions of 95 per cent alcohol and then with 4 or 5 small portions of ether, sucking dry after each addition of alcohol or ether. Add this amount of triple salt to a liter of acetone, shake, let stand overnight, and filter.

Standard Sodium Solution. Exactly 1 g. of c.p. sodium chloride is dissolved in water and made up to a liter in a volumetric flask. Each ml. of this solution contains 0.393 mg. of sodium.

ide. Add approximately 50 ml. of water free from carbon dioxide and 0.5 ml. of 1 per cent phenolphthalein solution and titrate with 0.02 N NaOH to a just barely perceptible pink, with a microburet graduated in 0.02 ml. A blank should be run to determine the amount of 0.02 N NaOH which will just give the end point with distilled water. Determine sodium on 0.5 ml. of standard sodium solution to standardize the procedure (see footnote 272, p. 651) and to facilitate determining the end point.

CALCULATION. According to Weinbach, the equation for the titration reaction is:

$$(UO_2)_3ZnNa(CH_3COO)_9 + 8NaOH \rightarrow 3(UO_2)(OH)_2 + Zn(OH)_2 + 9CH_3COONa$$

Hence 8 moles of NaOH are required for each mole of Na.[273] Each ml. of 0.02 N NaOH is thus equivalent to $(0.02 \times 23)/8 = 0.0575$ mg. of Na in the sample. If 1 ml. of a 1:10 filtrate is used,

$$(\text{Titration} - \text{Blank}) \times 0.0575 \times 100/0.1 = \text{mg. sodium per 100 ml. blood or serum}$$

To convert mg. per cent sodium into milliequivalents per liter, divide by 2.3.

Interpretation. Normally, the range of serum or plasma sodium content is approximately 300 to 330 mg. per 100 ml., or 130 to 143 milliequivalents per liter.[274] The sodium content of red cells is much less, ranging from 4 to 16 milliequivalents per liter.[275] Whole-blood sodium determinations have little clinical value. Sodium is the chief base of the plasma; its function there appears to be primarily physicochemical, in connection with the maintenance of osmotic pressure and acid-base balance. The organism has such a strong tendency to maintain a constant level of total base content that only slight changes are ordinarily found, even under pathological conditions; hence the need for precision in analyses. Significant decreases in plasma sodium content have been noted in pregnancy, in obstruction of the pylorus and other parts of the gastrointestinal tract, in pneumonia, in severe nephritis, and in Addison's disease. In the latter case, the decrease in the blood sodium level brought about by withdrawal of salt from the diet is of diagnostic significance.

DETERMINATION OF POTASSIUM

Introduction. Potassium, like sodium, is found in blood entirely in the form of the potassium ion. The distribution of blood potassium between cells and plasma is just opposite to that of sodium, potassium being much more abundant in the cells. Whole-blood potassium determinations have relatively little clinical significance; hence plasma or serum is ordinarily used for analysis. In obtaining plasma or serum, hemolysis must be avoided and separation from the cells should be carried out as soon as possible to minimize error due to diffusion of potassium from cells to surrounding fluid. Weichselbaum, Somogyi, and Rusk[276] advise that a syringe be dispensed with in obtaining blood, and allow the blood to flow directly from the needle in the vein into a small test tube, followed by standing for not over 20 minutes to permit clotting, and centrifuging to obtain the serum. The determination of potassium is usually based upon precipitation as the insoluble chloroplatinate[277] or cobaltinitrite[278] followed by titrimetric or colorimetric[279] estimation. Titrimetric and gravimetric methods based upon precipitation as the insoluble phospho-12-tungstate salt have also been described.[280]

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[273] According to Dregus, the titration yields sodium uranate, $Na_2U_2O_7$, zinc acetate, and sodium acetate at the end point, and 9 moles of NaOH are required instead of 8 for each mole of sodium present. To eliminate uncertainty, the best procedure is to determine the sodium equivalent of the sodium hydroxide by analysis of a standard sodium solution, and to use this factor in the calculations.

[274] Values are about 10 per cent higher if expressed in terms of a liter of plasma *water*

[275] Snyder and Katzenelbogen: *J. Biol. Chem.*, **143**, 223 (1942).

[276] Weichselbaum, Somogyi, and Rusk: *J. Biol. Chem.*, **132**, 343 (1940).

1. Determination by Flame Photometry: Principle. See p. 649, "Determination of Sodium." Since the potassium content of serum or plasma is much lower than the sodium content, the sample is ordinarily diluted 1:100 instead of 1:250. In the analysis of other tissues where the potassium content may be high and variable, suitable dilutions of the sample are usually established by trial.

Procedure. **Dilute 1 ml. of serum or plasma to 100 ml. with water and mix. Continue with the analysis as described on p. 650 for sodium. If an internal standard is used, add the required aliquot of stock solution to the volumetric flask containing the sample, before diluting to the 100 ml. mark. Ordinarily, the same concentration of internal standard is used for both sodium and potassium analyses; because of the lower total volume of diluted sample used in potassium analyses, proportionately less of the internal standard stock solution is required.**

STANDARDS. Standard solutions covering a range from 3 to 6 milliequivalents of potassium per liter, at a dilution of 1:100, are usually adequate for calibration purposes. Prepare a stock standard containing 10 milliequivalents of potassium per liter by dissolving 0.746 g. of KCl in water and diluting to 1 liter. Working standards equivalent to 3.0, 4.0, 5.0, and 6.0 milliequivalents of potassium per liter, at a dilution of 1:100, are prepared by diluting respectively 3.0, 4.0, 5.0, and 6.0 ml. of stock solution to 1 liter with water. If the analytical procedure requires an internal standard such as lithium sulfate solution, add to these working standards 10 times the volume of such solution routinely added to the diluted plasma or serum before diluting to the 1-liter mark and mixing. Prepare a calibration curve from these standards as described for the determination of sodium.

Interpretation. See under 2, below.

2. Method of Looney and Dyer:[281] Principle. Potassium is precipitated from the protein-free, chloride-free serum filtrate as the insoluble potassium silver cobaltinitrite of the Breh and Gaebler procedure. The washed precipitate is decomposed by alkali to liberate the nitrite, which is then determined colorimetrically by an application of the Bratton-Marshall sulfonamide method (see p. 658). The method has the advantage of not requiring a preliminary ashing of the sample, and yields a stable color.

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[277] Shohl and Bennett: *J. Biol. Chem.*, **78**, 643 (1928). The chloroplatinate methods usually require a preliminary ashing; for the use of electrodialysis to avoid ashing, see Sobel, Hanok, and Kramer: *J. Biol. Chem.*, **144**, 363 (1942), and other references cited for the determination of sodium by electrodialysis (p. 650). Micro-methods include those of Cunningham, Kirk, and Brooks: *J. Biol. Chem.*, **139**, 21 (1941), and Clark, Levitan, Gleason, and Greenberg: *ibid.*, **145**, 85 (1942). These methods are applicable to small amounts of tissue.

[278] Breh and Gaebler: *J. Biol. Chem.*, **87**, 81 (1930). This method is described in the eleventh edition of this book. See also Weichselbaum, Somogyi, and Rusk: *loc. cit.*

[279] Albanese and Wagner: *J. Lab. Clin. Med.*, **30**, 280 (1945).

[280] Van Slyke and Rieben: *J. Biol. Chem.*, **156**, 743 (1944); Rieben and Van Slyke: *ibid.*, **156**, 765 (1944).

[281] Looney and Dyer: *J. Lab. Clin. Med.*, **28**, 355 (1942).

Procedure.[282] Transfer 0.5 ml. of serum to a small clean test tube containing 7 ml. of distilled water, add 1 ml. of 1.5 per cent sodium tungstate, mix by tapping, and follow with 1 ml. of 2.5 per cent copper sulfate solution. Stopper and shake well; then add 0.5 ml. of 2.5 per cent silver nitrate solution. Stopper and shake again; then allow to stand 15 to 20 minutes. Pour onto a small dry filter (Whatman No. 5 is recommended), returning the first portion of filtrate to the funnel to ensure obtaining a clear filtrate.

Transfer 3 ml. of the filtrate to a clean 15-ml. graduated conical centrifuge tube,[283] and in a second similar tube place 3 ml. of the standard potassium solution, containing 0.03 mg. of potassium. Add 1 ml. of 95 per cent alcohol and 1 ml. of distilled water to each tube, mix by tapping, and place in a water bath at 18° to 22° C. for five minutes. Add 2 ml. of the freshly prepared and filtered silver cobaltinitrite reagent, mix by tapping, and replace in the water bath. Allow to stand 2 hours, then centrifuge for 15 minutes at 2800 r.p.m. Carefully remove the supernatant fluid down to the 0.2-ml. mark with a capillary pipet. Add 7 ml. of wash reagent down the sides of the tube, slanting the tube but disturbing the precipitate as little as possible. Again centrifuge for 15 minutes, decant the supernatant fluid, invert the tube, and allow to drain on filter paper for 5 minutes. Wipe excess fluid from the mouth of the tube, and repeat the washing and draining twice more.

To the washed precipitate in the centrifuge tube, add 10 ml. of 0.2 normal sodium hydroxide, breaking up the precipitate by blowing the alkali in or by tapping the tube. Place in a boiling water bath for 10 minutes. Remove, cool, and make up to 10 ml. with water. Mix well and centrifuge. Transfer 2 ml. of supernatant to a 100-ml. volumetric flask. Add 5 ml. of water, followed by 1 ml. of 50 per cent hydrochloric acid, and 2 ml. of 0.5 per cent sulfanilamide solution. Mix by lateral shaking, allow to stand 3 minutes, then add 1 ml. of the naphthylethylenediamine reagent. Dilute with water to the 100-ml. mark, and allow to stand 5 minutes before reading in the

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[282] Reagents Required: The sodium tungstate, copper sulfate ($CuSO_4 \cdot 5H_2O$), and silver nitrate solutions are prepared on the indicated basis from reagent-grade chemicals. Keep the silver nitrate solution in a brown bottle.

Standard Potassium Solution. Prepare a stock standard by dissolving 2.229 g. of pure dry potassium sulfate in water in a 1-liter volumetric flask, dilute with water to the mark, and mix. Preserve with a little toluene. This solution contains 1 mg. of potassium per ml. and is stable indefinitely. Prepare a working standard fresh daily by diluting 1 ml. of stock standard to 100 ml. with water in a volumetric flask. This solution contains 0.03 mg. of potassium in 3 ml.

Silver Cobaltinitrite Reagent. Prepare a sodium cobaltinitrite solution as follows: (A) Dissolve 25 g. of crystalline cobalt nitrate in 50 ml. of water, and add 12.5 ml. of acetic acid. (B) Dissolve 120 g. of reagent-grade sodium nitrite in 180 ml. of water. Add 210 ml. of B to all of A, place in the hood, and blow air through the solution until all of the nitrous oxide fumes have been driven off. This solution is stable for about 1 month if stored in the refrigerator. It must be filtered each time before using. To prepare the silver cobaltinitrite reagent, add 1 ml. of 40 per cent silver nitrate solution to 20 ml. of filtered sodium cobaltinitrite solution, shake well and filter. Prepare fresh for each series of analyses.

Wash Reagent. Mix 2 volumes of 95 per cent ethyl alcohol with 1 volume of ether and 2 volumes of water.

50 Per Cent Hydrochloric Acid. Mix 1 volume of water and 1 volume of concentrated hydrochloric acid.

Sulfanilamide Solution. Dissolve 0.5 g. of pure sulfanilamide powder (*not* the tablets) in a mixture of 30 ml. of glacial acetic acid and 70 ml. of water. Prepare fresh weekly.

Naphthylethylenediamine Reagent. Dissolve 0.1 g. of N-(1-naphthyl)-ethylenediamine dihydrochloride (obtainable from Eastman Kodak Co., Rochester, N.Y.) in a mixture of 30 ml. of acetic acid and 70 ml. of water. Prepare fresh weekly.

[283] Tubes should be cleaned with bichromate-sulfuric acid cleaning mixture and rinsed well with distilled water just before using. The presence of even traces of ammonia must be avoided, since ammonia likewise forms an insoluble cobaltinitrite.

colorimeter or photometer. For photometric measurement, set the photometer to zero density at 520 mμ with a blank prepared by treating 2 ml. of water in a 100-ml. volumetric flask by the procedure described above for 2 ml. of supernatant from the alkali treatment.

Calculation. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.03 \times \frac{10}{3} \times \frac{100}{0.5} = \text{mg. potassium per 100 ml. serum}$$

The standard corresponds to a serum potassium of 20 mg. per cent, covering the range from 10 to 40 mg. per cent satisfactorily. To convert mg. per cent potassium into milliequivalents per liter, divide by 3.9.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.03 \times \frac{10}{3} \times \frac{100}{0.5} = \text{mg. potassium per 100 ml. serum}$$

At 520 mμ, and in a 1-cm. cuvette, the standard has a density of approximately 0.160, and the agreement with Beer's law is such that up to 35 mg. per cent may be accurately determined. For higher values, or for photometric measurement at greater depth of solution, develop the color on 1-ml. instead of 2-ml. portions of the final standard and unknown supernatants. No change in calculations is involved.

Interpretation. The potassium concentration of human blood serum or plasma is relatively constant, ranging from about 16 to 22 mg. per cent, or 4.1 to 5.6 milliequivalents per liter. The potassium content of red cells is from 15 to 20 times as great as that of plasma. This indicates the essential uselessness of whole blood potassium determinations, depending as they must largely on the red cell count, and also emphasizes the importance of preventing hemolysis in obtaining serum or plasma for potassium analyses. The potassium content of plasma gradually increases on standing in contact with red blood cells; this is of importance in connection with the storage of whole blood in blood banks. Pathologically, increased serum potassium has been noted in acute bronchial asthma, in uremia, and in Addison's disease. Decreases have been noted at the time of seizure in familial periodic paralysis. The role of potassium in plasma is unknown; the plasma level may reflect to some extent the level in the body tissues and cells where potassium is abundant, being in fact the chief base present. In the body tissues and cells potassium appears to be the physiological equivalent of the plasma sodium in osmotic pressure regulation and acid-base balance.

DETERMINATION OF IRON

Introduction. The iron-containing compounds of biological material may be divided into two main groups, heme compounds and nonheme compounds. Heme iron is found in such substances as hemoglobin, cytochrome, catalase, etc., where it is firmly bound in organic combination with the porphyrin nucleus (see Chapter 22) and does not react with iron reagents until after liberation from such combination. Nonheme iron on the other hand appears to be largely extractable from tissues by suitable means and can be determined directly in the extracts as inorganic iron. There appears to be a rough correlation between the extractable iron of tissues and the fraction of the total iron which is absorbable and utilizable by the animal.[284]

Total iron is determined after wet or dry ashing, the iron being estimated in the ash solution by titrimetric[285] or colorimetric methods. Of the many color reagents for iron, the best appear to be thiocyanate,[286] thioglycollic (mercaptoacetic) acid,[287] *o*-phenanthroline,[288] 2,2′-bipyridyl ("α, α'-bipyridine"),[289] and protocatechuic acid.[290]

The total iron of blood is present almost entirely in the form of hemoglobin; the amount present will therefore vary with the hemoglobin content, and may be used as a measure of the latter (see p. 617). The amount of nonhemoglobin iron in blood is a matter of dispute, particularly with regard to that portion which is said to be in the red cells. According to Shorland and Wall,[291] the total nonhemoglobin iron of human blood is somewhat under 1 mg. per 100 ml. of whole blood, corresponding to about 2 per cent of the total blood iron; other investigators give higher values. Blood plasma contains about 0.1 mg. per cent of iron;[292] plasma iron may be of significance in iron transport and metabolism.

Determination of Total Iron in Blood, Foods, and Other Biological Material (***Methods of Elvehjem and of Kennedy***)***: Principle.*** The material is oxidized by ignition or by wet ashing. The acid solution is made alkaline and boiled to change pyrophosphate to orthophosphate. Thiocyanate is added, the ferric thiocyanate extracted with amyl alcohol[293] and determined colorimetrically. The method is suitable for substances relatively high in phosphate (such as milk) and for most biological material. For substances high in iron (as blood) the original procedure of Kennedy or the method of Wong may be used (see p. 617).

Method of Elvehjem.[294] **Ash a sample of the dried material containing 0.1 to 0.3 mg. of iron.**[295] **A few drops of nitric acid may be used to get rid of last traces of carbon. Dissolve by digesting with diluted HCl (1:1) at near the boiling point for 15 minutes or longer. Cool, make distinctly alkaline with 40 per cent NaOH (iron-free) and boil for 1 hour. Make acid with about 5 ml. of concentrated HCl and dilute to a volume of 50 ml. To 1 ml. of standard solution of iron**[296] **(equivalent to 0.1 mg. of Fe), add 5 ml. of concentrated HCl and dilute to 50 ml. Measure 10 ml. portions of standard and unknown**

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[284] Kohler, Hart, and Elvehjem: *J. Biol. Chem.*, **113**, 49 (1936). Their procedure for the determination of available iron is described in the eleventh edition of this book. See also Borgen and Elvehjem: *J. Biol. Chem.*, **119**, 725 (1937).

[285] Klumpp: *J. Biol. Chem.*, **107**, 213 (1934); Johnson and Hanke: *ibid.*, **114**, 157 (1936).

[286] See pp. 617 to 619. Also Thompson: *Ind. Eng. Chem., Anal. Ed.*, **16**, 646 (1944).

[287] See p. 618. Also Koenig and Johnson: *J. Biol. Chem.*, **142**, 233 (1942); Swank and Mellon: *Ind. Eng. Chem., Anal. Ed.*, **10**, 7 (1938).

[288] Saywell and Cunningham: *Ind. Eng. Chem., Anal. Ed.*, **9**, 67 (1937); Hummel and Willard: *ibid.*, **10**, 13 (1938); Fortune and Mellon: *ibid.*, **10**, 60 (1938); Bandemer and Schaible: *ibid.*, **16**, 317 (1944).

[289] Jackson: *Ind. Eng. Chem., Anal. Ed.*, **10**, 302 (1938); Moss and Mellon: *ibid.*, **14**, 862 (1942); Koenig and Johnson: *J. Biol. Chem.*, **143**, 159 (1942).

[290] Pereira: *J. Biol. Chem.*, **137**, 417 (1941).

[291] Shorland and Wall: *Biochem. J.*, **30**, 1049 (1936).

[292] Barkan and Walker: *J. Biol. Chem.*, **135**, 37 (1940). For a study of the determination of plasma iron, see Kitzes, Elvehjem, and Schuette: *J. Biol. Chem.*, **155**, 653 (1944).

[293] Thompson (*loc. cit.*) recommends extraction with isobutyl alcohol.

[294] Elvehjem: *J. Biol. Chem.*, **86**, 463 (1930). For iron content of animal tissues, see Elvehjem and Peterson: *J. Biol. Chem.*, **74**, 433 (1927).

[295] Farrar (*J. Biol. Chem.*, **110**, 685 (1935)) dusts tissue materials with iron-free calcium carbonate prior to ashing, in order to avoid loss of iron as chloride by volatilization.

[296] *Standard Iron Solution.* See p. 617.

into 50-ml. glass-stoppered cylinders. Add to each 10 ml. of amyl alcohol and 5 ml. of 20 per cent potassium thiocyanate. Shake thoroughly. Remove the colored layers of amyl alcohol with a pipet and compare in the colorimeter or determine the densities of standard and unknown in a photometer at 480 mμ. Set the photometer to zero density with a blank prepared as follows: dilute 5 ml. of concentrated HCl to 50 ml. with water, mix, and treat a 10-ml. portion with amyl alcohol and thiocyanate, shaking as described for standard and unknown. Remove the amyl alcohol layer and use as a photometric blank.

Method of Kennedy.[297] **Transfer 1 ml. of blood or a weighed piece of tissue to a 100-ml. Kjeldahl flask. Add 5 ml. of concentrated H_2SO_4 and 2 ml. of 60 per cent perchloric acid. Digest over a low flame until solution is colorless and dense fumes of SO_3 come off (about 10 minutes). Perhydrol may be used instead of perchloric acid. Cool, add a drop of nitric acid, and dilute to 100 ml. Carry out the same procedure on standard iron solution. Determine iron in 10-ml. aliquots exactly as in the preceding method.**

Calculation. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. Fe in standard} \times \frac{100}{\text{aliquot used}} = \text{mg. iron per 100 g. or ml.}$$

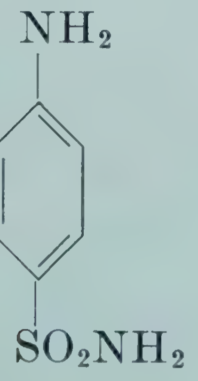
For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. Fe in standard} \times \frac{100}{\text{aliquot used}} = \text{mg. iron per 100 g. or ml.}$$

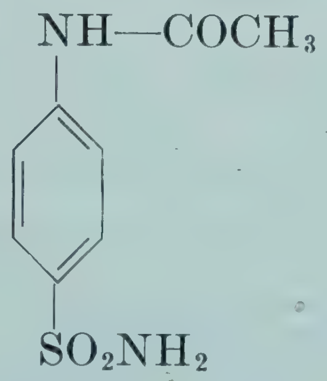
The photometric characteristics of the thiocyanate color in amyl alcohol are similar to those already given in connection with the Wong blood iron method (p. 618). Any of the other color reagents for iron which have already been mentioned (thioglycollic acid, *o*-phenanthroline, etc.) may be used instead of thiocyanate, under the proper conditions. For details, see original papers.

DETERMINATION OF SULFONAMIDES ("SULFA DRUGS")

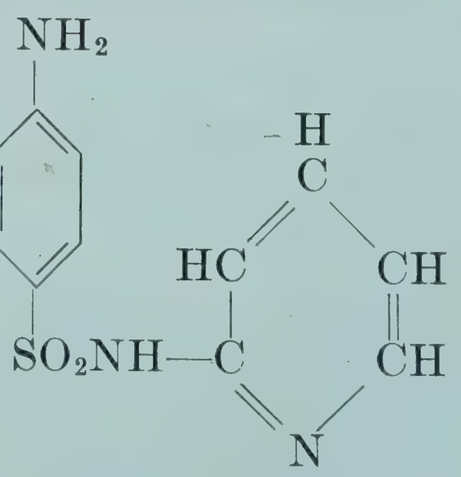
Introduction.[298] The "sulfa drugs" in clinical use are derivatives of the parent compound, sulfanilamide or *p*-aminobenzenesulfonamide. The structures of sulfanilamide, acetylsulfanilamide, and sulfapyridine are shown here, representing the general structures of the many "sulfa" drugs.



Sulfanilamide



Acetylsulfanilamide



Sulfapyridine

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[297] Kennedy: *J. Biol. Chem.*, **74**, 385 (1927).

[298] For historical reviews, see Krantz: *J. Am. Dent. Assoc.*, **31**, 634 (1944); Fosbinder: *J. Am. Pharm. Assoc.*, **33**, 1 (1944).

No sulfonamides are found in blood or urine unless they have been administered; the level attained depends upon such factors as the size and rate of administration of the dose, the body weight of the individual, and the rates of absorption and elimination. The therapeutically effective level depends upon both the disease and the drug; blood analysis permits definition of blood sulfonamide concentration so that a given level may be altered or maintained as conditions warrant. A variable portion of the total blood sulfonamide, the exact amount depending largely upon the type of sulfonamide being used, is usually present as the acetylated derivative[299] (e.g., acetylsulfanilamide). Acetylation involves the amino group attached to the benzene ring. Marked differences exist between the free and the acetylated compound in respect to both therapeutic and toxic properties; distinction is therefore important. Acetylation appears to occur largely in the liver. Other metabolic derivatives of certain of the sulfonamides are known, such as hydroxy derivatives, possibly combined with glucuronic acid;[300] the significance of these is somewhat obscure.

Determination of Sulfonamides (Method of Bratton and Marshall):[301] ***Principle.*** Blood is deproteinized with trichloroacetic acid. The protein-free filtrate is treated with nitrous acid to diazotize any free sulfonamide present, excess nitrous acid is destroyed, and the diazotized sulfonamide is coupled with N-(1-naphthyl)-ethylenediamine to form a stable red color which is then compared with that developed in a standard treated in the same way. The procedure is the same for all the common sulfonamides which have a diazotizable amino group in the molecule, the only difference being that a standard containing the particular sulfonamide being determined is used in each case. Since acetylation renders the sulfonamide incapable of diazotization, only the unacetylated sulfonamide will react in the colorimetric procedure. To determine acetylated sulfonamide, total sulfonamide is determined after acid hydrolysis, which frees the amino group. The difference between free and total sulfonamide represents acetylated sulfonamide.

Procedure.[302] **Measure 2 ml. of oxalated blood into a small flask and from a**

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[299] Marshall, Bratton, and Litchfield: *Science*, **88**, 597 (1938).

[300] Scudi: *Science*, **91**, 486 (1940); *also* Scudi and Jelinek: *J. Pharmacol.*, **81**, 218 (1944).

[301] Bratton and Marshall: *J. Biol. Chem.*, **128**, 537 (1939).

[302] Reagents Required: *Saponin Solution.* Dissolve 0.5 g. of saponin in water and dilute to 1 liter. This solution is not absolutely necessary, the saponin merely serving to hasten hemolysis of the red cells. The blood may be laked with water instead of saponin solution, in which case the diluted blood is allowed to stand 15 minutes before adding the trichloroacetic acid.

15 Per Cent Trichloroacetic Acid. Dissolve 150 g. of reagent-grade trichloroacetic acid in water and dilute to 1 liter. Stable indefinitely. To prepare a 3 per cent solution from this, dilute 1 volume with 4 volumes of water.

Sodium Nitrite Solution. Dissolve 0.1 g. of pure sodium nitrite in water and dilute to 100 ml. Prepare fresh daily.

Ammonium Sulfamate Solution. Dissolve 0.5 g. of ammonium sulfamate in water and dilute to 100 ml. Stable indefinitely.

N-(1-Naphthyl)-ethylenediamine Solution. Dissolve 0.1 g. of N-(1-naphthyl)-ethylenediamine dihydrochloride (obtainable from Eastman Kodak Co., Rochester, N.Y.) in water and dilute to 100 ml. Store in a dark glass bottle in the cold. Prepare fresh each week.

4 N Hydrochloric Acid. Dilute 40 ml., of concentrated hydrochloric acid to 100 ml. with water. Mix and titrate a 5-ml. portion with standard 1 N sodium hydroxide. Adjust to exactly 4 N. Stable indefinitely.

Standards. Prepare a *stock standard* as follows: dissolve in water exactly 0.1 g. of the particular sulfonamide being determined, and transfer with rinsings to a 1-liter volumetric flask. Dilute to the mark with water, and mix. In preparing this standard, use the pure pow-

buret add 30 ml. of saponin solution. Allow to stand for several minutes, then add 8 ml. of 15 per cent trichloroacetic acid solution. Mix well, allow to stand 5 minutes, and pour onto a dry filter.

Free Sulfonamide. **Transfer 10 ml. of the protein-free filtrate to a small flask or wide test tube, add 1 ml. of sodium nitrite solution, and mix. Allow to stand 3 minutes, then add 1 ml. of ammonium sulfamate solution and again mix. After 2 minutes' standing, add 1 ml. of the N-(1-naphthyl)-ethylenediamine solution and mix. The color develops almost immediately and is stable for several hours if not exposed to direct sunlight. Read the color in a colorimeter or photometer as described below.**

Total Sulfonamide. **Transfer 10 ml. of the protein-free filtrate to a test tube or other container graduated at 10 ml. and add 0.5 ml. of 4 N hydrochloric acid. Place in a boiling water bath for 1 hour, cool, and adjust the volume to 10 ml. with water. Treat with sodium nitrite solution, etc., just as described above for free sulfonamide.**

CALCULATION.[303]

For colorimetric measurement, compare the color developed on the unknown with that obtained by treating a 10-ml. portion of standard solution in the same manner as the unknown. Since the concentration of sulfonamide in blood may vary widely, several standards should be prepared and the unknown matched against the most suitable one. Satisfactory standards contain 1.0, 0.5, and 0.2 mg. per cent of the sulfonamide. At a blood dilution of 1:20, these standards correspond to blood levels of 20, 10, and 4 mg. per cent respectively. It is convenient to set the 1-mg. standard at 10 mm. in the colorimeter, the 0.5 mg. standard at 15 mm., and the 0.2 mg. standard at 20 mm.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 20 \times \text{mg. per cent concentration of standard}$$
$$= \text{mg. per cent free (or total) sulfonamide in blood}$$

For photometric measurement, transfer the colored solution to a suitable container and read in a photometer at 530 $m\mu$.[304] Set the photometer to zero density with a blank obtained by treating a 10-ml. portion of 3 per cent trichloroacetic acid with nitrite, sulfamate, etc., as in the treatment of the unknown. From the photometric density of the unknown, and the density of a known standard, preferably determined at the same time, calculate as follows:[303]

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 20 \times \text{mg. per cent concentration of standard}$$
$$= \text{mg. per cent free (or total) sulfonamide in blood}$$

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dered drug, *not* the tablets. This standard is stable for months in the cold. To prepare *dilute standards* containing 1.0, 0.5, and 0.2 mg. per cent, measure 10, 5, and 2 ml. respectively of the stock standard into 100-ml. volumetric flasks containing 18 ml. of 15 per cent trichloroacetic acid. Dilute with water to the 100 ml. mark and mix. Store in the cold, and prepare fresh every few days.

[303] With some of the sulfonamides, correction factors are necessary to correct for drug lost during the precipitation of the proteins. With sulfanilamide, sulfaguanidine, sulfapyridine at levels less than 5 mg. per cent, and free sulfathiazole, no correction factors are needed. For sulfapyridine levels greater than 5 mg. per cent, multiply the results for both free and total drug by 1.1 to obtain the correct values. For acetylated sulfathiazole, subtract the uncorrected free value from the uncorrected total value and multiply the difference by 1.3.

[304] Filters or wavelength settings from 520 to 540 $m\mu$ give equally satisfactory results (see Fig. 167).

The density of a standard containing 0.2 mg. per cent sulfonamide in a 1-cm. cuvette is approximately 0.300 at 530 mμ (Fig. 167). This means that at this solution thickness, bloods containing up to about 10 to 12 mg. per cent sulfonamide may be read satisfactorily, since agreement with Beer's law is excellent over a wide range. Higher concentrations produce colors which are too deep for precise measurement, and in such cases the determination must be repeated at a greater dilution of the blood (or of the 1:20 filtrate, diluted with 3 per cent trichloroacetic acid). If a solution thickness greater than 1 cm. is used in the photometer, the range of reading is proportionately reduced. If some dilution other than 20 is used, this dilution should replace the 20 in the above calculations. Bratton and Marshall point out that dilutions of 1:50 or 1:100 may be employed in this procedure if photometric measurement is used, thus permitting the use of fingertip blood (0.1 to 0.2 ml.). The only precaution is that the final concentration of trichloroacetic acid in the protein-free aliquot taken for analysis should be 3 per cent.

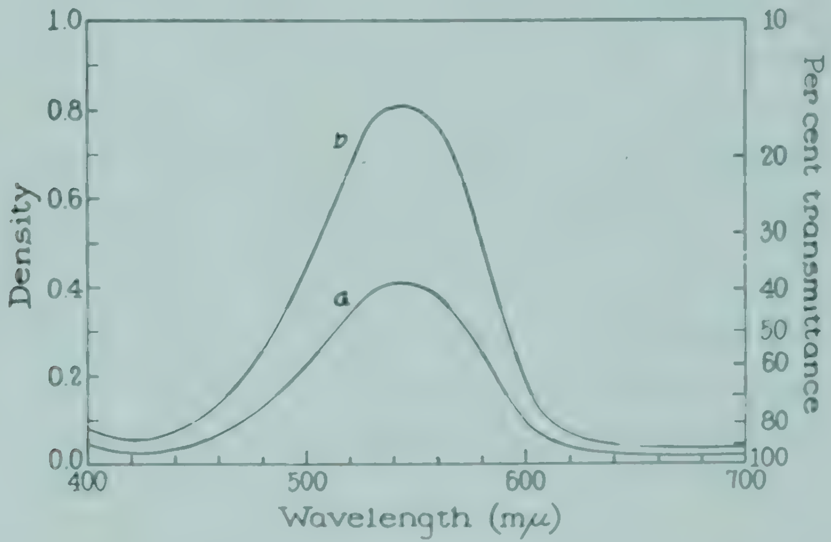

Fig. 167. Absorption Spectra of Colored Solutions Obtained in Bratton-Marshall Sulfonamide Method.

For standards containing (*a*) 0.25 mg. per cent, and (*b*) 0.50 mg. per cent sulfadiazine. Solution depth, 1 cm.

Interpretation. The major factors influencing the level of blood sulfonamide content have already been presented (p. 658); depending upon circumstances, the total blood sulfonamide concentration may range from a trace to as high as 20 mg. per cent or more, with a variable proportion of this in the acetylated form. It is the level of *free* drug which is of therapeutic importance; it is usually considered clinically that effective levels of free sulfonamide range from 3–7 mg. per cent for sulfathiazole to as high as 15 mg. per cent for sulfanilamide and sulfadiazine. Those metabolic derivatives other than acetylated forms which have thus far been recognized do not appear to require hydrolysis before reacting in the colorimetric procedure, and will therefore be included in the value for free sulfonamide content; the extent to which this occurs, and the precise significance of these derivatives has not as yet been evaluated.

DETERMINATION OF PROTEIN-BOUND IODINE

Method of Chaney:[305] ***Principle.*** The iodine-containing hormones (thyroxine and triiodothyronine), along with other organic compounds containing iodine, if present, are precipitable with serum proteins by tungstic acid, zinc hydroxide, etc.

By digestion of the protein precipitate with chromic-sulfuric acids, the iodine is oxidized to iodic acid which remains in the acid digest. Other halides and volatile products of oxidation are distilled off during the digestion.

On adding an excess of reducing agents, the iodic acid is reduced, and iodine distilled off in a special still and collected in dilute alkali and arsenite. The concentration of iodine in the distillate is measured by its catalytic effect on the rate of reduction of ceric salts by arsenious acid. This may be done by a series of colorimeter readings at 5-minute intervals, or by a continuous recording colorimeter. The method is suitable for determination of trace quantities of iodine in serum, tissue, or other biological material, in the range of 0.01 to 1 μg.

Procedure. **The proteins in 3 ml. of serum or plasma are precipitated by 30 ml. of tungstic acid (1 volume ⅔ N sulfuric acid, 1 volume 10 per cent sodium tungstate, 16 volumes water), or 24 ml. of zinc sulfate (1.25 per cent) and 3.0 ml. of sodium hydroxide (0.75 N) in a 50-ml. centrifuge tube, and the clear supernatant liquid is tested by the catalytic method (see below) for the presence of interfering quantities of inorganic iodide. If the serum contains more than 25 μg. of inorganic iodine per 100 ml., additional washing of the protein precipitate is required.**

The protein precipitate is then dissolved in 20 ml. of sulfuric acid (78 per cent by weight and iodine-free) and transferred with the aid of 15 ml. additional sulfuric acid to a 300-ml. two-necked digestion flask containing 2 ml. of chromic acid (concentration 100 g./100 ml.). Digestion is carried out for a period of about 10 minutes over a 350 watt electric heater till the temperature reaches about 230° C. The digest is allowed to cool, 15 ml. of water added, and the flask reheated to a temperature of 200° C.

After cooling and diluting with 20 ml. of water, the flask is attached to the special still and condenser and each of the following added through a side tube, to reduce iodic acid and the excess chromic acid in the digest: 1 ml. 8 M phosphorous acid, 1 ml. 0.45 N arsenious acid and 1 ml. 1.5 per cent hydrogen peroxide. To the still trap is added 1 ml. of 1 N potassium hydroxide and distillation is carried out for 6 minutes. The distillate in the trap is run into a tube calibrated at 12 ml., containing 0.25 ml. of 0.15 N arsenious acid, and diluted to volume.

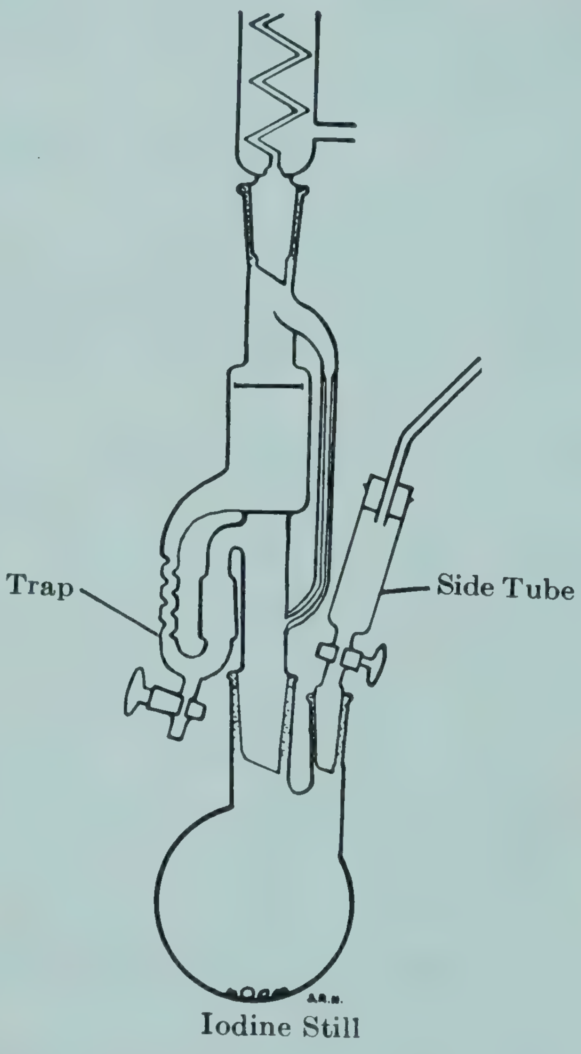

FIG. 168. IODINE DISTILLATION APPARATUS.
Courtesy, Dr. Albert L. Chaney.

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[305] Chaney: *Ind. Eng. Chem. Anal. Ed.*, **12**, 179 (1940); Taurog and Chaikoff: *J. Biol. Chem.*, **163**, 313 (1946); Barker: *J. Biol. Chem.*, **173**, 715 (1948); Connor, Curtis, *et al.*: *Surgery*, **25**, 510 (1949); Kydd, Man, and Peters: *J. Clin. Invest.*, **29**, 1033 (1950); Chaney: *Anal. Chem.*, **22**, 939 (1950).

The iodine concentration in the distillate is measured by taking a 4 ml. aliquot in a colorimeter tube, adding 0.5 ml. of 0.15 N arsenious acid, and 0.5 ml. of 0.10 N ceric sulfate in 3.5 N sulfuric acid, and measuring the rate of color fading at a constant temperature (30° C.), and comparing this rate with that of known quantities of iodine (0.01-0.6 μg.) diluted with similar amounts of alkali, arsenious acid, and ceric sulfate. Color measurements are made at 5-minute intervals, using a blue filter (480 mμ), and the rate of color change calculated or plotted.

Reagents should be specially selected and purified if necessary so that iodine content of quantities required for a complete determination does not exceed 0.03–0.05 μg.

Interpretation. Normal serum protein-bound iodine values range from 4 to 8 μg. per 100 ml. In hyperthyroidism, values range from 8 to 30 μg. per 100 ml., while in hypothyroid states, levels are 0 to 4 μg./100 ml. Low values are also sometimes found in nephrosis and other conditions with very low serum proteins. In pregnancy a mild elevation of protein-bound iodine occurs. False high values may occur following administration of "Lipiodol," x-ray diagnostic agents ("Priodax," "Neo-Iopax," "Skiodan"), "Itrumil," and "Diodoquin."

CLINICAL MICROCHEMICAL ANALYSIS

The following discussion of clinical microchemical analysis is a brief introduction to the field, offered to the reader to encourage him to venture in and apply his ingenuity in solving problems related to the analysis of small amounts of biological fluids. Brevity precludes adequate

Scales of Measurement in Microchemical Analysis, Terminology, and Examples in Clinical Chemistry

Quantity Measured	*Abbreviations*	*Current Terminology*	*Suggested Terminology*	*Examples in Clinical Chemistry Currently Used*
20 Milligrams	mg.	Semimicro	Milligram chemistry	Macro-Kjeldahl analysis for nitrogen
1 Milligram*	mg.	Micro	Milligram chemistry	Micro-Kjeldahl analysis for nitrogen
1 Microgram	μg., gamma, or γ	Ultramicro	Microgram chemistry	Fluorimetric analysis for urinary coproporphryrins
0.001 Microgram	mμg.	Submicro	Millimicrogram chemistry	Catalytic analysis for protein-bound iodine
0.000001 Microgram	μμg.	Micromicro	Micromicrogram chemistry	Microbiological assay for vitamin B_{12}
1 Milliliter†	ml.			
1 Microliter	μl., lambda, or λ			

* 1 gram = 1,000 milligrams = 1,000,000 micrograms.

† 1 liter = 1,000 milliliters = 1,000,000 microliters.

recognition of the numerous original workers who did the pioneering. The definition of microchemical analysis is difficult because of the prevalence of numerous descriptive terms. The table on page 662 contains some of the terms, abbreviations, and usages. The examples of analytical microtechniques used in clinical chemistry reflect the range of measurements encountered. Where concentrations are high (e.g., protein in plasma ≐ 70,000 μg./ml.), minute volumes may be used for analysis; however, where low (e.g., protein-bound iodine in plasma = 0.05 μg./ml.), larger volumes must be used.

It should be noted that many clinical chemical determinations of necessity are microtechniques, because they deal with trace amounts of material. For example, quantities as small as 0.5 μg. of coproporphyrin in 5 ml. of urine, 0.1 μg. of tryptophan in 0.02 ml. serum, or 0.05 μg. of iodine in 1 ml. serum may be concerned in a routine analysis. These figures indicate that minuteness of sample volume is not a consistent guide to the minuteness of substance measured.

The advantages of microchemical procedures in clinical chemistry are numerous. For example, where serial chemical data on infants are desired, standard procedures and their required volumes of blood are impractical. Sobel[306] and Natelson[307] have developed routine procedures requiring but a few drops of blood, readily obtained from the fingertip or heel of the infant. Lowry, Bessey, and co-workers developed micromethods for mass nutritional surveys, using fingertip blood for serum proteins,[308] alkaline phosphatase,[309] vitamin A,[310] vitamin C,[311] iron,[312] riboflavin,[313] tocopherols,[314] and thiamine.[315]

In addition to savings in equipment (syringes, glassware, reagents, and laboratory, refrigerator, and centrifuge space) it was possible to make great savings in time and perform these analyses at the commendable rate of 50 per hour. Blood chemistry in small animals also requires microtechniques.

Certain characteristics of microchemical analysis are uniquely favorable. For example, dry ashing of 10 μl. of serum takes a few minutes, whereas larger amounts require considerably more time. In the analysis for calcium, if proteins are to be removed, ashing is easier than protein precipitation and collection of the supernatant fluid. In analyses using microdiffusion for separation, the rates of diffusion can be increased by reducing the reaction volumes.

Some disadvantages are the special requirements of apparatus, skills, and methodology. Kirk[316] has pointed out, however, that there is no sacrifice of accuracy in microchemistry.

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[306] Sobel and Hanok: *Mikrochemie ver. Mikrochim. Acta*, **39**, 51 (1952).
[307] Natelson: *Am. J. Clin. Path.*, **21**, 1153 (1951).
[308] Lowry and Hunter: *J. Biol. Chem.*, **159**, 465 (1945).
[309] Bessey, Lowry, and Brock: *J. Biol. Chem.*, **164**, 321 (1946).
[310] Bessey, Lowry, Brock, and Lopez: *J. Biol. Chem.*, **166**, 177 (1946).
[311] Bessey, Lowry, and Brock: *J. Biol. Chem.*, **168**, 197 (1947).
[312] Burch, Lowry, Bessey, and Berson: *J. Biol. Chem.*, **174**, 791 (1948).
[313] Burch, Bessey, and Lowry: *J. Biol. Chem.*, **175**, 457 (1948).
[314] Quaife, Scrimshaw, and Lowry: *J. Biol. Chem.*, **180**, 1229 (1949).
[315] Burch, Bessey, Love, and Lowry: *J. Biol. Chem.*, **198**, 477 (1952).
[316] Kirk: *Quantitative Ultramicroanalysis*, New York, John Wiley & Sons, Inc., 1950.

No new physicochemical laws apply to microchemistry. In classical methods requiring about 1 millimole of material, 6×10^{20} molecules are measured; in microchemistry about 6×10^{15} molecules are measured, which is still a large number of molecules. This implies that reactions which take place stoichiometrically with large quantities of substance ought to work with smaller amounts, and in practice, almost all good clinical chemical procedures can be performed on smaller amounts of material. Most of the facilities for measurement are limited by final concentrations of the desired substance. A major device, then, in microchemistry is to limit the volume of the final reaction mixture. For example, in the titration of 50 ml. of 0.01 N HCl with 0.01 N NaOH using methyl red as the indicator, 100 ml. of final reaction mixture is obtained at the end point. With the addition of 0.05 ml. (0.1 per cent of the titrating agent) a visible change in end point occurs. By reducing the volume from 100 ml. to 0.1 ml., an increment of 0.1 per cent of the titrating agent should visibly affect the end point. If there is no sacrifice in accuracy of measurement, the analysis is reduced from the measurement of 500 μeq. to 0.5 μeq. with no loss in sensitivity at the small scale of analysis. The same is true for colorimetry. For example, 50 μl. of normal blood (1.0 ml. of 1:20 filtrate) containing about 50 μg. of glucose, when analyzed for glucose, will give sufficient blue color in a final volume of 25 ml. to be measured in any colorimeter. Reducing the reaction mixture to 1 ml. and measuring the color in a suitable colorimeter allows the measurement of 2 μg. of glucose (equivalent to 2 μl. of blood).

Other approaches include the use of special sensitive measurements such as fluorimetric, microbiologic, catalytic, enzymatic, spectrophotometric, and other methods of analysis which use specific chemical, biological, or physical properties of the substance being measured. The possible adaptations to microclinical chemistry are innumerable and are facilitated by the ingenuity of the analyst and good equipment.

APPARATUS AND TECHNIQUES

Precise microweighing in clinical chemistry is possible but not essential. Measurements involving microprecipitates are made colorimetrically or titrimetrically. For example, calcium precipitated as the phosphate is measured colorimetrically, and calcium precipitated as the oxalate is measured titrimetrically. Most standards are weighed on ordinary analytical balances and diluted to suitable volumes.

Trace materials can usually be isolated, purified, and concentrated by chromatography (resin, paper, silica gel, alumina, etc.) or countercurrent distribution (see Chapter 1). Special methods may require special equipment which can either be made by the analyst or purchased.

Micropipets. Several excellent micropipets[317] have been described and successfully used. Three are shown in Fig. 169. The pipet (*A*) of Sisco, Cunningham, and Kirk,[318] which delivers from 1 to 1000 μl. is widely used and readily available commercially. The straight tube pipet (*B*) is simple, is easy to construct and calibrate, and can deliver accurately volumes of 1 to 1000 μl. or more. *C* represents a self-filling pipet used for automatically measuring 1 to 10 μl. This type may also be used as an overflow pipet. The constriction-type pipet made by Levy[319] is a very convenient micropipet delivering from 1 to 500 μl. when properly made and used. Levy,[319] Lowry,[309] and Natelson[307] provide details of construction. As an alternative, straight-tube blowout pipets (Fig. 169, *B*) with fine tips may be used. When rinsed with water or reaction mixture, they will deliver contained volumes with errors of 0.1 to 0.5 per cent. Pipets of over 20 μl. when emptied by drainage alone will deliver volumes with 1 per cent error or less. The error is smaller in the larger pipets. Siliconed pipets deliver the contained volume without drainage error;[320] hence they can achieve excellent replicability down to 0.1 per cent error. Furthermore, the complete drainage eliminates the need for rinsing between samples. This advantage fails with whole blood but holds with blood filtrates. Pipets are calibrated to contain or to deliver, depending upon their use. The delivered or contained (as desired) weight of water is a convenient basis for calibrating pipets. The weight of water in mg. × 1.004 (at 25° C.) gives the volume in μl. Mercury weight (in mg.) delivered × 0.0739 is the volume in μl. Other convenient methods involve measuring the delivered quantity of chloride, acid, or dye from a solution of known concentration. Unsiliconed pipets used to deliver should be emptied slowly and for the length of time specified in the calibration, and then the remaining contents should be emptied by gently blowing while the pipet is rotated slowly to transfer the tip contents onto the wall of the container. A fine tip allows excellent replication.

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[317] Microchemical Specialties Co., 1834 University Ave., Berkeley, Calif., lists about 12 types of micropipets ranging in volume from 0.25 to 1000 μl.

[318] Sisco, Cunningham, and Kirk: *J. Biol. Chem.*, **139**, 1 (1941).

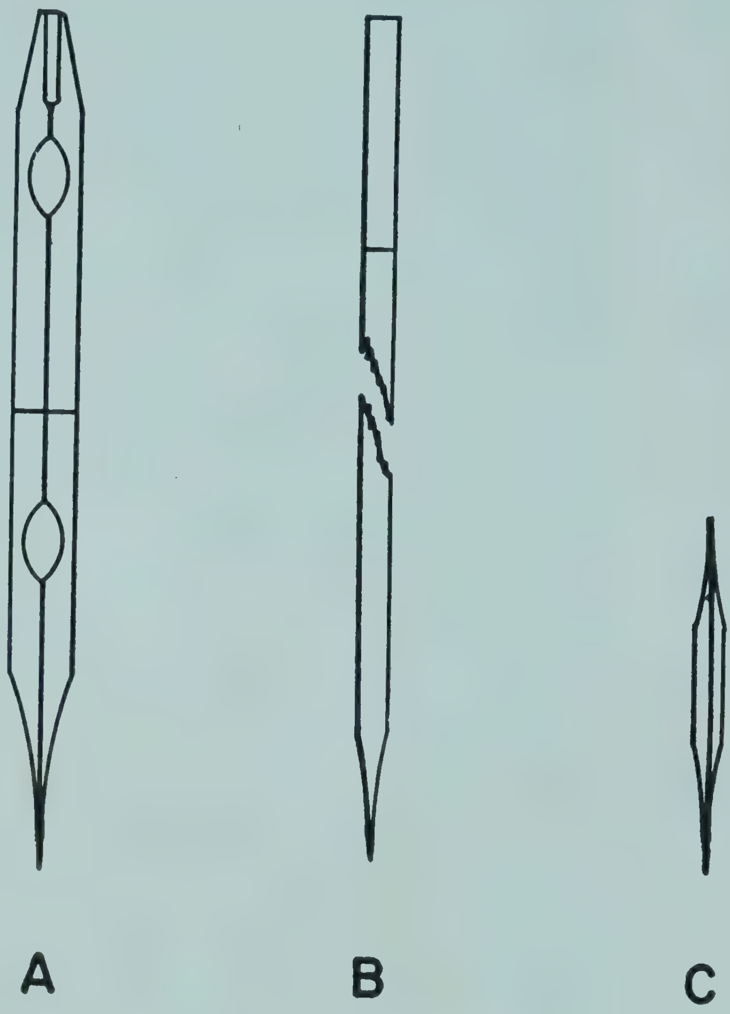

FIG. 169. MICROPIPETS.

A, Pipet of Sisco, Cunningham, and Kirk; *B*, straight tube pipet; *C*, self-filling or overflow pipet, depending upon how it is used.

Microburets. There are a number of suitable, ingenious microburets described in the literature, many of which are made commercially. Especially satisfactory is the Scholander[321] type, an example of which is shown in Fig. 170. This buret was made from a machinist's 1-inch micrometer, which can be obtained with a scale calibrated in very small fractions of a millimeter or inch. As the micrometer is turned clockwise it propels the spindle into the mercury as shown in Fig. 170 and displaces a volume equivalent to the volume of the shaft that enters the mercury, simultaneously displacing an equal volume of the standard solution above the mercury. A fine opening in the buret tip prevents back diffusion of the reaction mixture into the buret. A titration is made to the desired end point, whereupon a reading of the micrometer is made and a new titration is begun. The tip is not washed between titrations. After the buret is emptied, more standard reagent is added by turning the micrometer handle counterclockwise. The tip should be wide enough to allow rapid filling without pulling in air through the joint. The usual micrometer will deliver about 30 μl. per mm. (or 75 μl. per 0.1 inch) and the buret will deliver this volume to ±0.03 μl. or 0.1 per cent. For ordinary work one filling of standard solution will provide for 8 to 25 continuous titrations. Smaller deliveries with equal precision can be achieved by reducing the diameter of the shaft which enters the mercury.

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[319] Levy: *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **21**, 101 (1936).
[320] Duggan and Smith: *Science*, **116**, 305 (1952).
[321] Scholander: *Science*, **95**, 177 (1942).

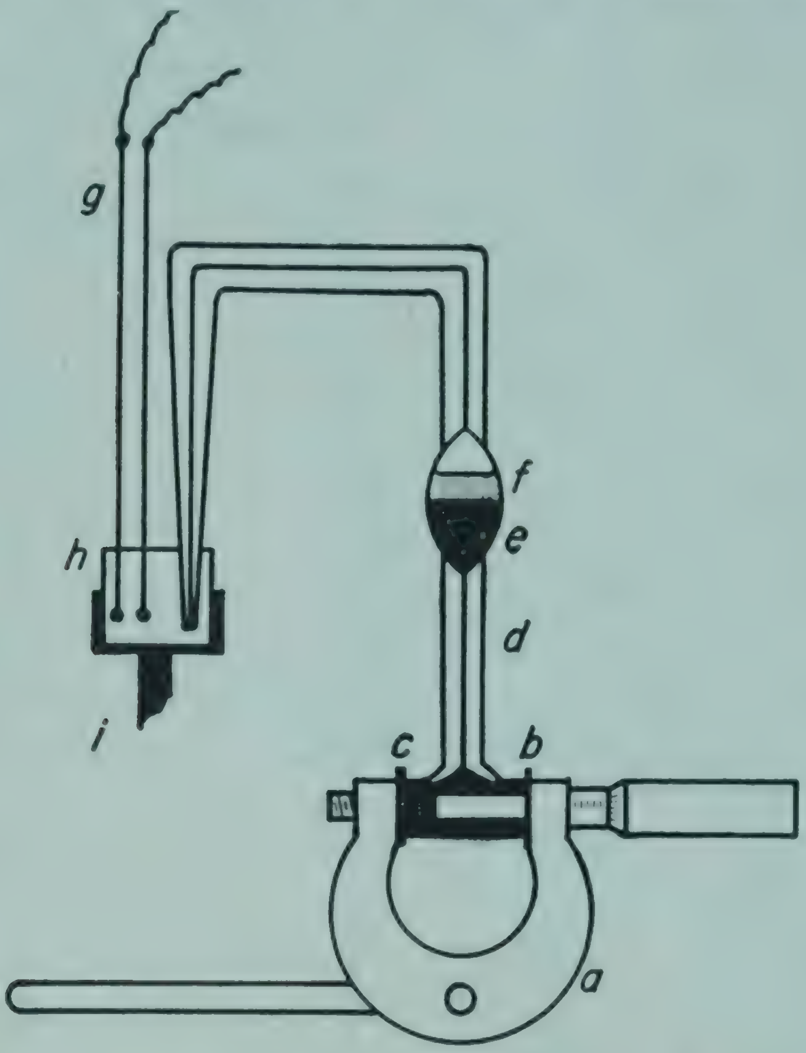

FIG. 170. SCHOLANDER MICROBURET AS USED WITH THE MICROTITRATION TABLE SHOWN IN FIG. 171.

The setup shown is for the electrometric determination of chloride: *a*, 1-inch micrometer and rod used for holder; *b*, gasket; *c*, set screw and metal plate; *d*, glass buret; *e*, mercury; *f*, carbon tetrachloride; *g*, special electrodes; *h*, 15 × 15 mm. vial which holds 1 ml. of reaction mixture; *i*, vial-holder attached to shaft of titrator.

The mercury displacement buret has been criticized on three points:

(1) expansion with heat, (2) leaks through couplings, and (3) reaction of mercury with reagents above it. Expansion errors are negligible when wide temperature fluctuations are avoided. Leaks are rare in properly maintained burets. The problem of reagent interaction with mercury is completely eliminated for most aqueous reagents by interposing a 3 to 5 mm. (about 0.3 ml.) layer of carbon tetrachloride between reagent and mercury.

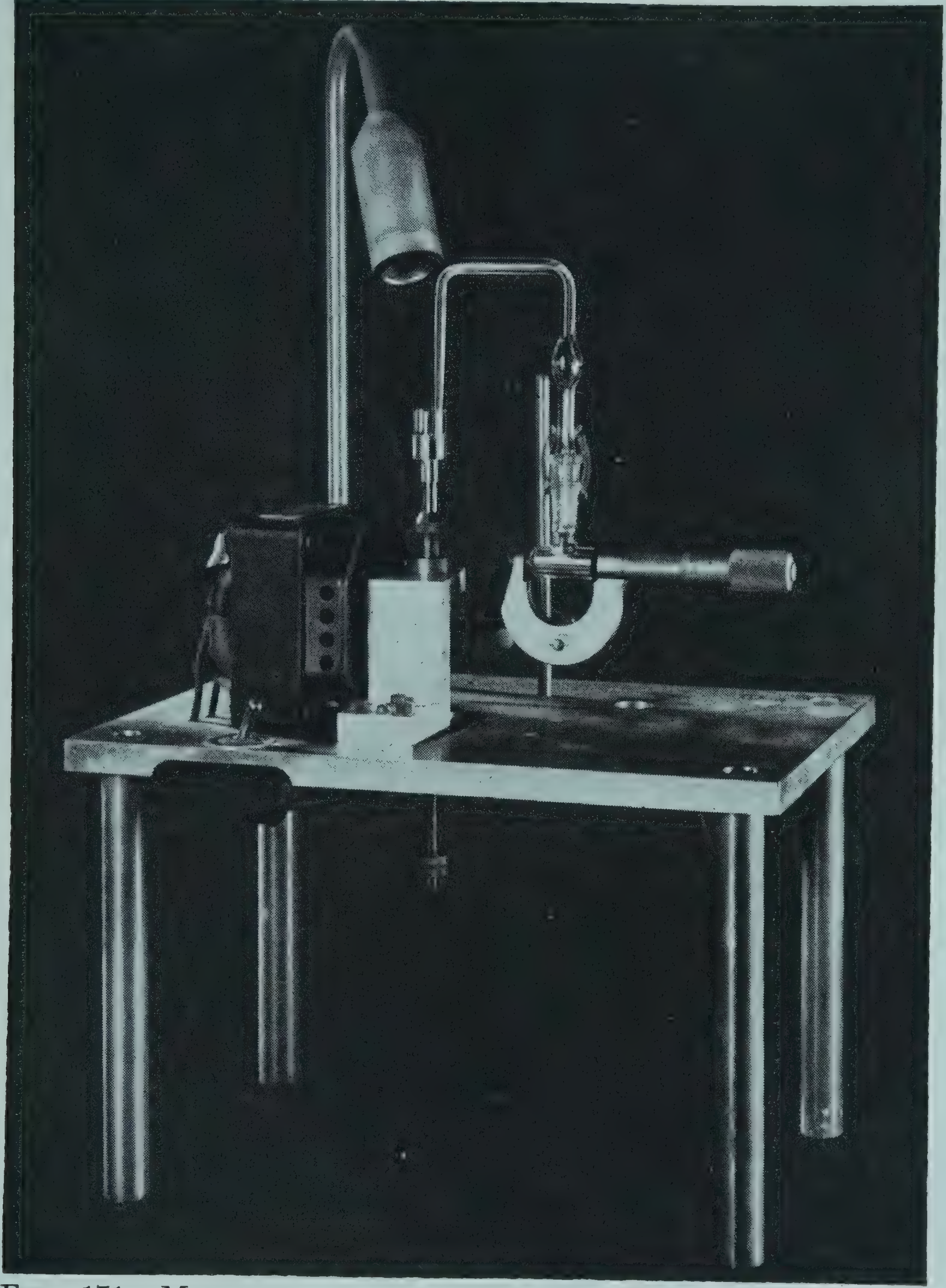

FIG. 171. MICROTITRATION TABLE WITH MOUNTED SCHOLANDER BURET.

The vial fits into a holder attached to the rotator. The latter can be raised and lowered. The buret tip functions as a stirring rod.

Courtesy, Walter Reed Army Medical Center, Washington, D. C.

A microtitration table,[322] devised so that the buret described here can readily be mounted, is shown in Fig. 171. When the vessel into which the buret tip is immersed is rotated rapidly the buret tip stirs the reaction mixture. Reaction vessels from 0.1 to 5 ml. may be used, and rotated at 300 to 900 r.p.m. Any sensitive indicator or special electrodes using standard equipment and circuits can be used for titrations. At the end point the rotating shaft is lowered, a new titration vessel is inserted into the holder, and the shaft is raised into position for the new titration. Glass vials of a suitable diameter are cut to vertical size for titration vessels. A white penicillin-bottle stopper[323] is suitable for holding volumes of 100 μl. Acidimetric titrations in 1-ml. volumes may be performed using a standard pH meter fitted with glass, quinhydrone, or antimony electrodes; the latter two kinds of electrodes have been used routinely in 100 μl. volumes. Titration curves of excellent quality and accuracy are obtained. The calomel half cell (see p. 47) is made from a sidearm test tube with the sidearm bent and drawn out to a fine tip. Agar is used to plug the tip, which is inserted into the titration vessel along with the opposite electrode and the buret tip.

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[322] Made by H. Kugler, College Park, Md.

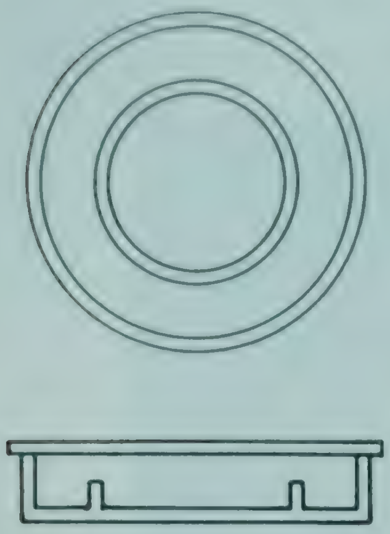

FIG. 172. DIAGRAM OF CONWAY DIFFUSION CELL.

Reaction Vessels. Titration vessels can be made from glass vials cut to a suitable size. Standard small test tubes of 3-ml. (10 × 75 mm.) or 1-ml. (6 × 50 mm.) volume serve as reaction vessels which can be heated, cooled or otherwise manipulated. Such test tubes can also be made into volumetric flasks. Small centrifuge tubes from 0.1 ml. up and high speed microcentrifuges are available commercially.

Microdiffusion Apparatus. The Conway cell[324] (Fig. 172) for diffusion has been well and universally studied and applied. In conventional use, the reaction mixture is placed in the outer chamber and the receiving solution in the inner chamber. The dish is sealed with a lubricated glass plate. Any liberated gas diffuses from the outer to the inner chamber. In the determination of NH_3, the inner chamber contains HCl or other suitable acid; for the determination of CO_2, the inner chamber contains $Ba(OH)_2$. The determination is made by titrating the solution in the inner chamber or withdrawing the contents for colorimetric analysis.

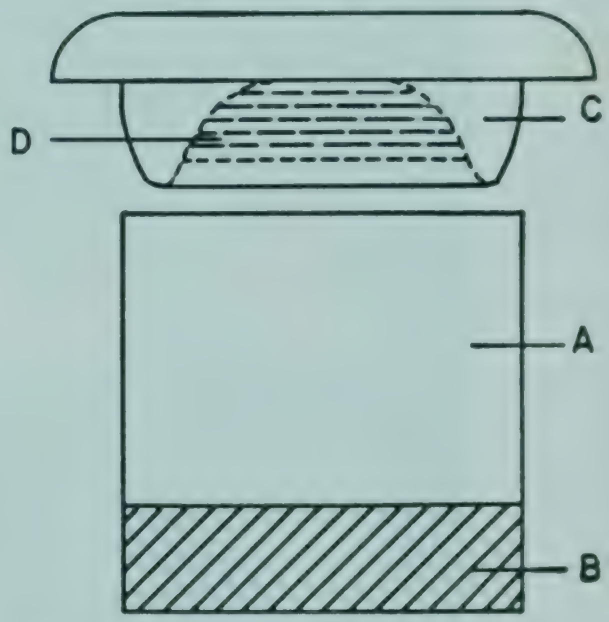

FIG. 173. MICRODIFFUSION CELL. *A*, 15 × 15 mm. vial; *B*, reaction mixture which liberates gas or absorbs it; *C*, rubber penicillin-bottle stopper; *D*, well in the stopper which contains solution that receives gas or liberates it. In the determination of CO_2, gas is liberated with lactic acid at *B* and received in $Ba(OH)_2$ at *D*. In the determination of ethanol, blood is placed at *D* and the liberated ethanol is oxidized by acid dichromate at *B*.

In Fig. 173 is shown a similar cell made from a 15 mm. × 15 mm. vial

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[323] Type S-46 obtained from T. C. Wheaton Co., Millville, N. J.

[324] Conway: *Microdiffusion Analysis and Volumetric Error*, London, Crosby Lockwood & Son, Ltd., 1950.

(*A*) to contain the reaction mixture, and a penicillin-bottle stopper (*C*) for the receiver. In the determination of bicarbonate,[325] acid is placed at *B*, and a measured quantity of $Ba(OH)_2$ is placed at *D*. The sample (blood) is introduced into the vial and the liberated CO_2 is received by diffusion into the $Ba(OH)_2$ above. The stopper (*D*) is inverted and placed on the titrator and the excess $Ba(OH)_2$ is measured acidimetrically. Another example of the use of this cell is in the measurement of blood alcohol where 50 to 100 μl. of blood are placed in stopper *D* and 0.500 ml. of standard acid dichromate in the vial at *B*. The diffused alcohol is measured by titrating the excess dichromate remaining after oxidation of the ethanol.

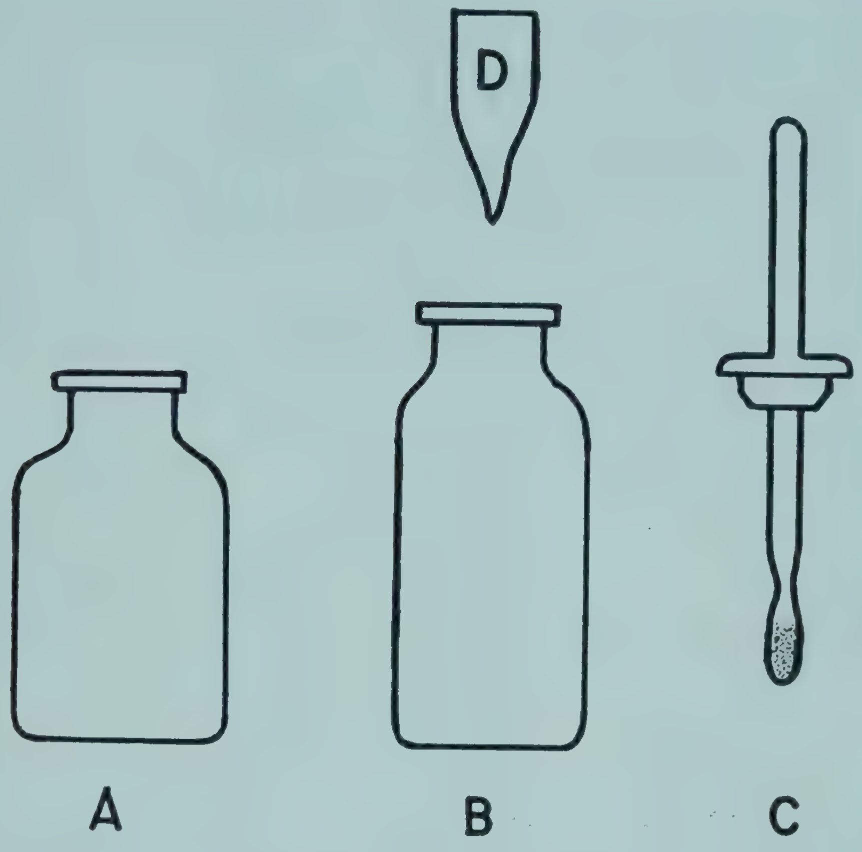

FIG. 174. DIFFUSION BOTTLES FOR THE DETERMINATION OF NH_3.

A, Penicillin-type bottle; *B*, streptomycin-type bottle; *C*, glass receiving rod, 6 × 90 mm., with a constriction 10–15 mm. from the end. The stippled area represents a roughened surface (ground) to aid in retaining the film of acid which receives the diffused NH_3. *D*, cap for bottle *B*.

Another group of diffusion cells[326] is shown in Fig. 174. *A* is a standard penicillin bottle.[327] *C* is a glass rod fitted into a penicillin-bottle stopper. The rod has a constriction at one pole. The tip surface is ground so that when inserted into acid it will retain a film of the acid. A sample containing NH_3 is introduced into *A*. The final volume is adjusted to 1.0 ml. The solution is made alkaline with K_2CO_3 crystals; the receiving rod, previously dipped into 1 N H_2SO_4 to the constriction, is inserted into *A* and closed. The entire cell may be placed in a rotator (Fig. 175) and rotated so that diffusion is accelerated. The NH_3 collected on the receiving rod is measured colorimetrically. *B* is a streptomycin[328] bottle in which a Kjeldahl determination may be carried out.

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[325] Seligson and Seligson: *Anal. Chem.*, **23**, 1877 (1951).

[326] Seligson and Seligson: *J. Lab. Clin. Med.*, **38**, 324 (1951).

[327] Type S-205, NO-SOL-VIT bottle, T. C. Wheaton Co., Millville, N. J.

Photometry. Any one of the several available photoelectric colorimeters may be used for microchemistry. The Lowry and Bessey adaptation for the Beckman spectrophotometer has the advantage that volumes down to 25 μl. can be examined over a wavelength range from 235 to 935 mμ. Microcuvettes[329] (3 × 10 × 25 mm. inside measurements) hold 0.8 ml. and may be used in some of the newer instruments without special carriers. For smaller volumes a sheet of metal with a 1.0–1.4 mm. hole narrows the beam of light so that it will pass through 100 μl. or less. Lowry states that rinsing between samples is often unnecessary in serial determinations of similar samples. Siliconed cuvettes eliminate the need for rinsing.

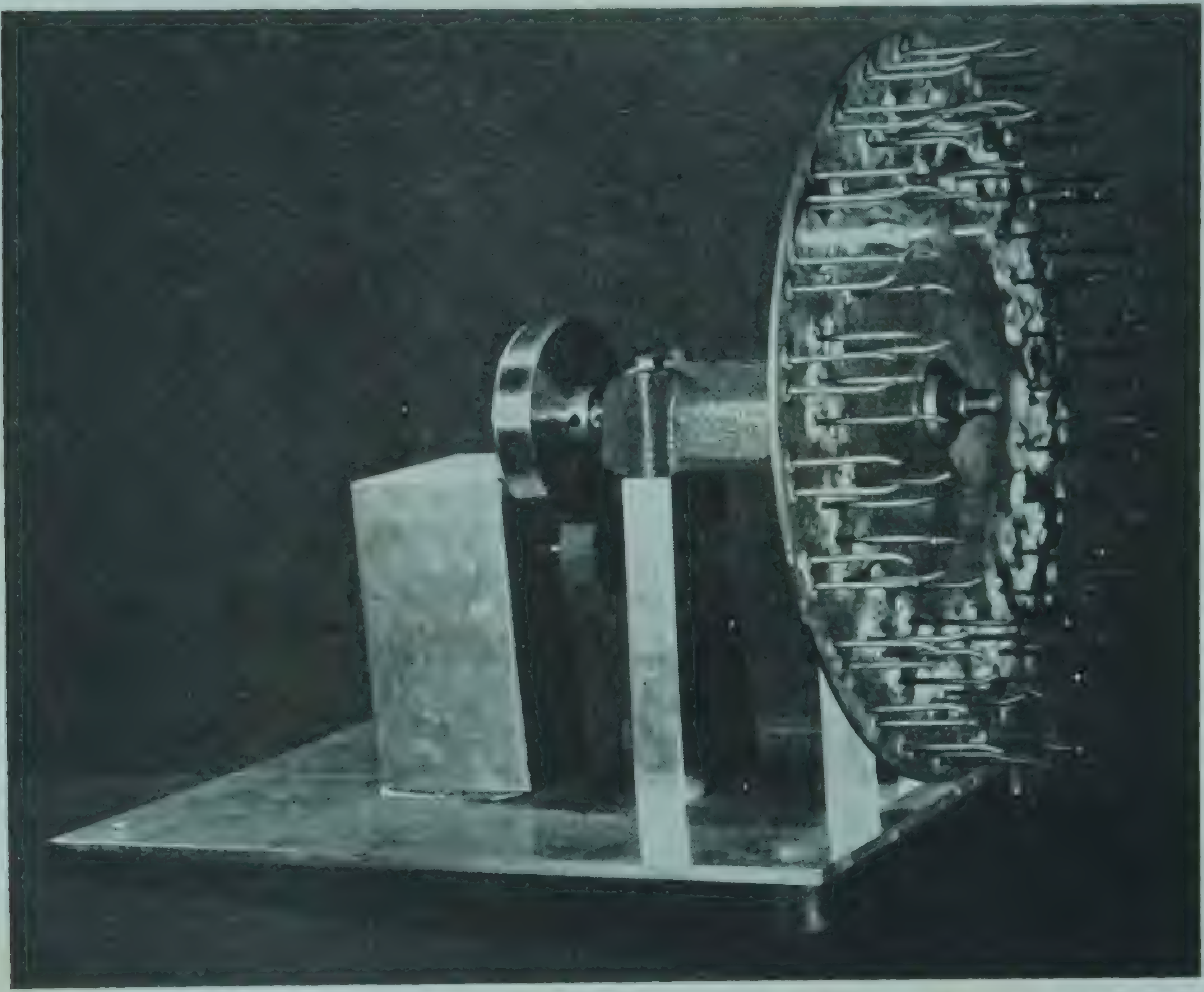

FIG. 175. ROTATOR FOR FACILITATING DIFFUSION OF NH_3 IN BOTTLES SHOWN IN FIG. 174.

Courtesy, Walter Reed Army Medical Center, Washington, D. C.

CLINICAL MICROCHEMICAL PROCEDURES

Determination of Urinary Ammonia Nitrogen:[326] ***Principle.*** Ammonium ions are converted to NH_3 by alkalinization; the liberated NH_3 gas is collected by diffusion and measured colorimetrically. The working range is 5 to 15 μg. of NH_3-N; it may be reduced to 0.5–1.5 μg. by suitable adjustments. This basic method is applicable to every system which liberates NH_3 stoichiometrically. For example, urease will hydrolyze urea, acid will hydrolyze amides (glutamine, urea, etc.), and ninhydrin will oxidize amino acids to liberate NH_3. The classical Kjeldahl method can also be applied as illustrated below.

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[328] Type S-281, NO-SOL-VIT bottle, T. C. Wheaton Co., Millville, N. J.

[329] Pyrocell Manufacturing Co., 207 E. 84th St., N. Y. C.

Procedure.[330] **Add 50 μl. of urine to a diffusion bottle (*A*, Fig. 174); add water to 1.0 ml. and 1 g. $K_2CO_3 \cdot 1\frac{1}{2}H_2O$. Stopper with the receiving rod (*C*, Fig. 174) previously dipped to the indentation in 1 N H_2SO_4. Place the bottle on the rotator (Fig. 175) and rotate for 30 minutes (complete diffusion). Transfer the rods to clean bottles or cuvettes and wash the acid into the bottle or cuvette with 10.0 ml. of diluted Nessler reagent. Read in 5 minutes at 420 mμ and compare with a standard (1.0 ml.) and a blank (1.0 ml. of water) treated as described for the diluted urine sample.**

If speed is essential, rotation may be stopped at 10 minutes and the incomplete diffusion (90 per cent) corrected by comparing unknowns to similarly run standards. If smaller amounts of Nessler reagent are used, the working range diminishes accordingly. One μg. of nitrogen per ml. of Nessler reagent is a good ratio. After diffusion the collected NH_3 on the receiving rod can be stored in a clean bottle and measured at the analyst's convenience. The error is 1–2 per cent. Where air contamination by NH_3 is a problem, equipment should be rinsed and dried before use.

Determination of Urea in Blood, Urine, Serum, or Other Biological Fluids:[331] ***Principle.*** The method above is applied by measuring the NH_3 liberated by the hydrolysis of urea with urease. The same apparatus is used.

Procedure. **Transfer 50 μl. of serum or 10 μl. of urine to a diffusion bottle, make to 1.0 ml. with 0.05 M phosphate buffer, pH 7.0, and add 1 drop of fresh 5 per cent urease suspension. After 15 minutes at 40° C., add 1 g. $K_2CO_3 \cdot 1\frac{1}{2}H_2O$ and cap with a stopper and receiving rod previously dipped in 1 N H_2SO_4. Rotate for 30 minutes. Measure the collected NH_3 as described above, comparing with NH_3 standards and a reagent blank. Correct urine for contained NH_3, determined as described above. Fresh blood or serum requires no correction. Measurements can be made on 5 μl. of sample if desired.**

Analyses of a sample of urea on 8 different days produced a standard error of 0.5 per cent. All the usual precautions for protecting urease apply.

Determination of Total Nitrogen by Kjeldahl Digestion and Microdiffusion:[326] ***Principle.*** The sample is digested with a concentrated acid digestion mixture until all of the nitrogen present has been converted into ammonia. The ammonia is released by alkali as usual, and collected by microdiffusion. The collected ammonia is determined colorimetrically. The method as described is for the determination of nonprotein nitrogen (NPN) in a protein-free plasma filtrate. It is equally applicable to the determination of total N in urine, plasma proteins, etc. The size of sample should be such as to contain not more than approximately 15 μg. of total N.

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[330] Reagents Required: *Standard* $(NH_4)_2SO_4$, *15 μg. N per ml.*
1 N H_2SO_4.
$K_2CO_3 \cdot 1\frac{1}{2}H_2O$ *Crystals.* Fit bottle with spoon to deliver about 1 g.
Nessler Reagent of Vanselow (*Ind. Eng. Chem., Anal. Ed.*, **12**, 516 (1940)). Dissolve 34.9 g. KI and 45.5 g. HgI_2 in as little water as possible. Add 112 g. KOH and water to 1 liter; let stand 3 days; dilute 1:5 before using.

[331] Seligson and Bollier: To be published.

Procedure.[332] A protein-free filtrate is obtained by adding 9 parts of 3.0 per cent trichloroacetic acid to 1 part of plasma, and filtering. Add 0.5 ml. of 1:10 filtrate (equivalent to 50 μl. of serum) or other sample to the hard glass bottle (*B*, Fig. 174) followed by 1.0 ml. of digestion mixture. Heat slightly on a hot plate to just short of boiling, and continue heating until all traces of water disappear from the bottle, as manifested by an absence of droplets at the sides. Slowly raise the hot-plate temperature to about 500° C. and add cap (*D*, Fig. 174) to prevent loss of spray. A suitable temperature is achieved when the sulfuric acid refluxes one-half way up the bottle. Heat for 2 hours. Cool and wash down caps and sides of flasks with 1.5 ml. of alkaline washing solution, using a fine-tipped pipet. Add 4 to 5 pellets of NaOH with tweezers and stopper with an acid-coated receiving rod (*C*, Fig. 174) as described on p. 671 for the determination of urinary ammonia. Rotate 90 minutes for complete diffusion. Wash the ammonia from the rod with 10.0 ml. of Nessler reagent and measure the color as described previously. Prepare standards and blanks as usual.

Determination of Chloride:[333] ***Principle.*** This is a modification of the Cunningham[334] procedure. The sample of plasma, urine, or other fluid is made strongly acid; the chloride is titrated with $AgNO_3$ until the special electrodes give off current indicating the appearance of Ag^+ and the disappearance of Cl^-.

Procedure.[335] Clean two electrodes[336] by repeated immersion in 10 per cent HNO_3. Amalgamate one electrode by repeated immersion in clean mercury beneath 10 per cent HNO_3. The silver electrode tip is stored in water, the amalgamated one in mercury. Place 50 μl. of serum or urine in a vial; add 2 drops of 3.6 N H_2SO_4. Place the vial on the titration assembly (Fig. 171); add water to about 1 ml. Arrange electrodes and microburet as shown in Fig. 170. The electrodes should reach to about 2 mm. from the bottom of the vial, and the vial should rotate at about 600–800 r.p.m. The wiring diagram is shown in Fig. 176. Adjust the galvanometer to zero with the aid of back current from the potentiometer. Add $AgNO_3$ from the buret until the galvanometer indicator moves to and remains for 15 seconds at a predetermined point on the scale. The buret and the contained $AgNO_3$ can be calibrated against standard chloride solution to give readings of chloride content directly. The end point is chosen as the inflection point of a titration curve made by plotting galvanometer units against buret units.

Titrations may be performed at the rate of one per minute with an error of less than 1 per cent. Equilibration is rapid because fast rotation of the vial brings the chloride, silver, and electrodes effectively together. Studies of the method reveal no interference from proteins, as shown by the agreement of results on protein-free filtrates, ashed serum, and whole serum. Urines analyzed directly and after ashing gave the same results.

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[332] Reagents Required: *Digestion mixture.* Dissolve 12 g. $HgSO_4$, 100 g. K_2SO_4, and 180 ml. H_2SO_4 in water and make up to 1 liter.

Washing Solution. Dissolve 50 g. $Na_2S_2O_3$ and 40 g. NaOH in water and make up to 1 liter.

NaOH pellets.

[333] Seligson, McCormick, and Sleeman: To be published.

[334] Cunningham, Kirk, and Brooks: *J. Biol. Chem.*, **139**, 11 (1941).

[335] Reagents Required: *0.1N* $AgNO_3$.

3.6 N H_2SO_4.

Standard Chloride Solution. 0.0100 N NaCl or HCl.

[336] *Silver electrodes.* 18-gauge pure silver wire, heated at one end to form a 1–2 mm. ball. Sterling silver has been successfully used

The method is adaptable to one μg. of chloride by reducing volumes, using smaller electrodes and more dilute solutions.

Determination of Blood Alcohol Content[337] ***(Modified from Winnick***[338]***):*** ***Principle.*** The alcohol is separated from the blood by microdiffusion and collected in standard chromic acid solution, where it is oxidized. The chromic acid remaining is titrated iodimetrically with thiosulfate. The thiosulfate is standardized by analyzing a standard alcohol solution.

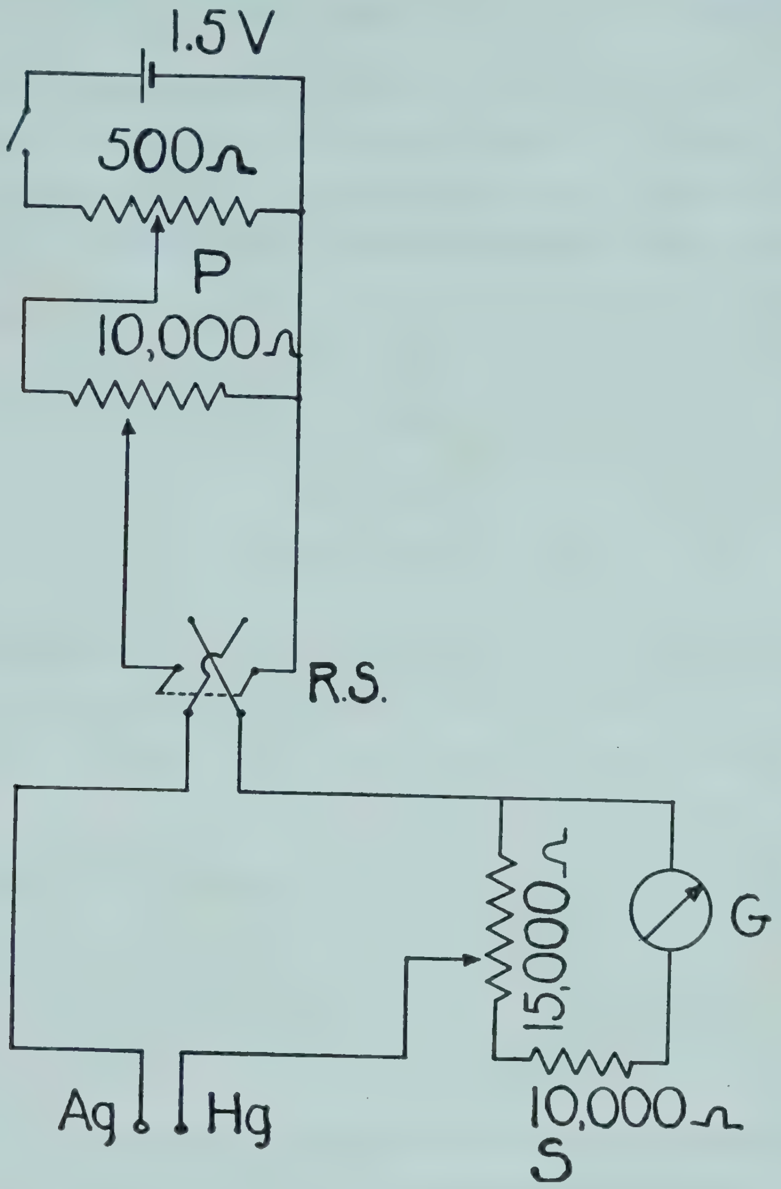

FIG. 176. CIRCUIT FOR MEASUREMENT OF CHLORIDE.

Ag, silver wire electrode; *Hg*, silver wire electrode amalgamated on its surface; *G*, galvanometer sensitive to 7.34×10^{-10} amp. (G.E. #13); *S*, shunt for the galvanometer; *R.S.*, reversing switch; *P*, potentiometer made from two variable resistors and a 1.5-volt battery. See Fig. 171 for electrode and microburet arrangement on the microtitration table.

Procedure.[339] **Blood is prevented from clotting by the use of sodium fluoride, and if not used may be kept in the refrigerator for 24 hours. Pipet 250 μl. of**

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[337] Seligson and Sleeman: Unpublished.

[338] Winnick: *Ind. Eng. Chem. Anal. Ed.*, **14**, 523 (1942).

[339] Reagents Required: *Standard Chromic Acid Solution.* $K_2Cr_2O_7$, 0.05 N in 10 N H_2SO_4.
0.2 *N* $Na_2S_2O_3$.
Potassium iodide. 20 per cent.
Starch Solution. 0.1 per cent.
$K_2CO_3 \cdot 1\frac{1}{2}H_2O$ C.P.
Standard Alcohol. 160 mg./100 ml. Dilute 2.00 ml. of absolute alcohol (sp. g. 0.800) to 1 liter with distilled water, and mix.

the standard chromic acid solution into a glass vial (*A*, Fig. 173). Pipet 50 μl. of blood into the well of the rubber stopper (*D*). Add about 0.1 g. K_2CO_3 (large crystals) to the well. When this is moistened by the blood, no carbonate will fall out. Stopper the vial and allow to stand overnight in an oven at 37° C., to allow diffusion and oxidation to become complete. Run a parallel determination on water alone (50 μl.) as a blank, and on 50 μl. of standard alcohol solution.

When diffusion is complete, remove the stoppers and titrate the chromic acid in each vial as follows: Add 1 drop of KI solution, place the vial on the titrating table, and titrate with standard (0.2 N) thiosulfate until the solution is faintly yellow. Add 1 drop of starch solution and titrate to the disappearance of the blue color.

Calculation. Subtract the buret units required for the standard from the value found for the blank. Call this value *a*. Subtract the buret units required for the sample from the value for the blank. Call this value *b*. Since the standard represents 160 mg. per cent alcohol, the calculation is as follows:

$$\text{mg. per cent alcohol in blood} = \frac{160}{a} \times b$$

The alcohol in as little as 10 μl. of blood can be measured by reducing the concentration of chromic acid and thiosulfate. Higher temperatures will accelerate diffusion and oxidation. The error of the analysis is approximately 3 per cent.

Microdetermination of Acid or Alkali: Principle. The solution to be titrated is placed in a titration assembly fitted with a suitable pair of electrodes (calomel and either glass, quinhydrone, or antimony[340] electrodes) attached to a pH meter. The pH is measured after each increment of added standard alkali or acid, as the case may be. Titration is continued to the desired pH end point.

Procedure. **Place 1.0 ml. of the solution to be titrated in a 15 × 17 mm. vial. Place in the holder of the titration table (Fig. 171) and insert the electrodes, which are connected to a pH meter. Start the rotating shaft and read the pH as increments of standard alkali (or acid) are added. Read the buret when the desired pH is reached.**

With this system, titrations can be carried out rapidly to the desired end point. For acid solutions approximately 0.01 N in strength, 0.2 N alkali is satisfactory. By reducing volumes and concentrations and employing antimony or quinhydrone electrodes, as little as 100 μl. of solution may be titrated. Errors due to contamination by the CO_2 of the air may be eliminated in most instances by blowing a gentle stream of CO_2-free air over the surface of the titrating vessel.

The procedure described here has been found particularly useful in microbiological assays based on measurement of acid production.[341] Titrations were carried out in a final volume of one ml., in 15 × 15 mm. vials which were incubated in a Petri dish modified to hold 25 vials. A spatula tip of quinhydrone was added to each vial contents, and titration continued to pH 7.0, using a platinum-calomel electrode system. With one filling of alkali in the buret, from 25 to 50 vials could be titrated at a rate of better than 1 per minute, with an accuracy exceeding the requirements of the method.

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[340] Levin: *Chemist-Analyst*, 41, 89 (1952).

[341] Seligson, Anderson, and Torghele: *J. Lab. Clin. Med.*, 35, 640 (1950).

Measurement of pH and Hematocrit. Scholander[342] and others have devised accurate microgasimetric methods for CO_2, N_2, O_2, and CO. For clinical purposes the method of Shock and Hastings (see p. 703) for pH, CO_2, and hematocrit is very convenient and desirable. Singer and Hastings[343] provide a helpful nomogram and discussion for evaluation of various forms of acidosis and alkalosis, using the data obtained by this method.

Measurement of Sodium and Potassium. Suitable but relatively tedious microchemical techniques have been established for the determination of sodium and potassium. With the advent of flame photometry, rapid, accurate micromeasurements are easily achieved (see pp. 650 and 653). Sodium (0.7 μeq.) in 2.5 ml. of 1:500 serum (5 μl.) can be measured in most available instruments. Potassium (0.1 μeq.) in 2.5 ml. of 1:100 serum (25 μl.) can similarly be measured, both with an error of 1 to 2 per cent. Increased sensitivity can be achieved by including acetone in the diluent.

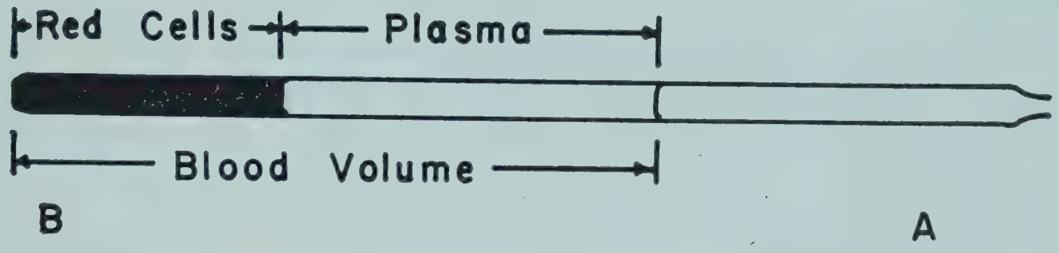

Fig. 177. Collection of Blood in a Melting-Point Tube.
A, Tube, 1.5–2.0 mm. O.D. × 100 mm. The end at *B* is sealed and the tube is centrifuged to pack the red cells. The hematocrit equals mm. packed red cells/mm. blood. Cutting the tube at the red cell–plasma interface allows exact separation of plasma for microanalyses.

Other Methods. Natelson[307] has described a system for measuring hematocrit, plasma Na, K, Cl, protein, urea and glucose on 100 μl. of fingertip blood. Blood is drawn by capillarity into a 1.5 × 11 mm. tube[344] (*ca.* 0.1 ml., Fig. 177), sealed at one end, and centrifuged at 4,000 r.p.m. for 10 minutes. The fractions are measured with a rule and the volume of packed red cells calculated. The tube is cut at the red cell–plasma junction and analyzed for the constituents named above. Sobel[345] has described a microtitration method for calcium which appears to be more reliable than micro flame-photometry methods. Total serum proteins and albumin-globulin measurements may be made by the biuret[346] colorimetric technique except that reaction volumes are reduced to meet the need of sample size. For example, 100 μl. of serum in a final volume of 10 ml. in the standard biuret procedure can be reduced to 10 μl. in a final volume of 1.0 ml. Similar reductions in any desired salting-out procedure can be made; however, the usual precautions concerning denaturation of albumin by excessive shaking in the salt mixture and ether, and the variation due to differences in salt mixtures, should be observed. Paper electrophoresis[347] (see p. 464) makes it possible to fractionate proteins in serum obtained from a few drops of fingertip blood.

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[342] Scholander, Flemister, and Irving: *J. Biol. Chem.*, **169**, 75 (1947).
[343] Singer and Hastings: *Medicine*, **27**, 223 (1948).
[344] Kimble 34500, wet with heparin and dried before use.
[345] Sobel and Sobel: *J. Biol. Chem.*, **129**, 721 (1939).
[346] Gornall, Bardawill, and David: *J. Biol. Chem.*, **177**, 751 (1949).

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[347] Levin and Oberholzer: *Am. J. Clin. Pathol.*, **23,** 205 (1953).

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(*Methods for the analysis of blood for bicarbonate content, blood gases, and hydrogen-ion concentration are given in Chapter 24. Methods of analysis for various vitamins will be found in Chapter 35, and for penicillin in Chapter 36.*)

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24

Respiratory Exchange and Neutrality Regulation

General. The major function of the lungs in respiration is to facilitate the addition of oxygen to the blood and to remove excess carbon dioxide from the blood. The oxygen is carried to all the tissues, where it is utilized in the metabolic processes taking place within the cells. The chief end products of these metabolic processes include H_2O, CO_2, urea, and various organic and inorganic acids such as lactic, uric, phosphoric, sulfuric, and the like. The production of end products which are bases, such as ammonia and the various organic amines, is relatively unusual or confined to lower species or to special tissues. Of the various end products listed, only H_2O and urea are neutral substances and can be excreted by the body without bringing into play the various mechanisms of neutrality regulation which are the subject of this discussion.

The CO_2 produced in the tissues is removed by diffusion into the blood, where the major portion (approximately 70 per cent) at once undergoes hydration to form carbonic acid, the remainder combining with the blood proteins (including hemoglobin) to form carbamates (20 per cent), or remaining in physical solution (10 per cent). The newly formed carbonic acid must be immediately neutralized or the blood would become far more acid than is compatible with life; this neutralization is mediated largely through hemoglobin, as described below. The other acid end products of metabolism are neutralized as soon as they are formed and exist in the blood and tissues as salts, neutralization being effected through reaction with such ions as the HPO_4^{--} ion largely in the cells and the HCO_3^- ion largely in the blood plasma. Thus carbonic acid is not only an important end product of tissue oxidation, but it also plays a significant role in neutrality regulation. The level of the blood bicarbonate content is the most satisfactory single index of the ability of the body as a whole to neutralize acid end products of metabolism; hence the term "alkali reserve." The peculiar virtue of the carbonic acid–bicarbonate system in controlling the neutrality of the body lies in the volatility of carbon dioxide and hence its ready elimination by the lungs. The nonvolatile acids (uric, phosphoric, etc.) after conversion into their salts can be disposed of by the kidneys.

Role of Oxygen. A complete understanding of the mechanism of neutrality regulation and CO_2 transport in the blood requires knowledge of the part played by hemoglobin in the transport of oxygen (see Fig. 178). Oxygen is found in the blood in two forms, (*a*) in physical solution, and (*b*) in combination with the respiratory pigment hemoglobin to give the compound oxyhemoglobin. The amount of oxygen in physical solution in

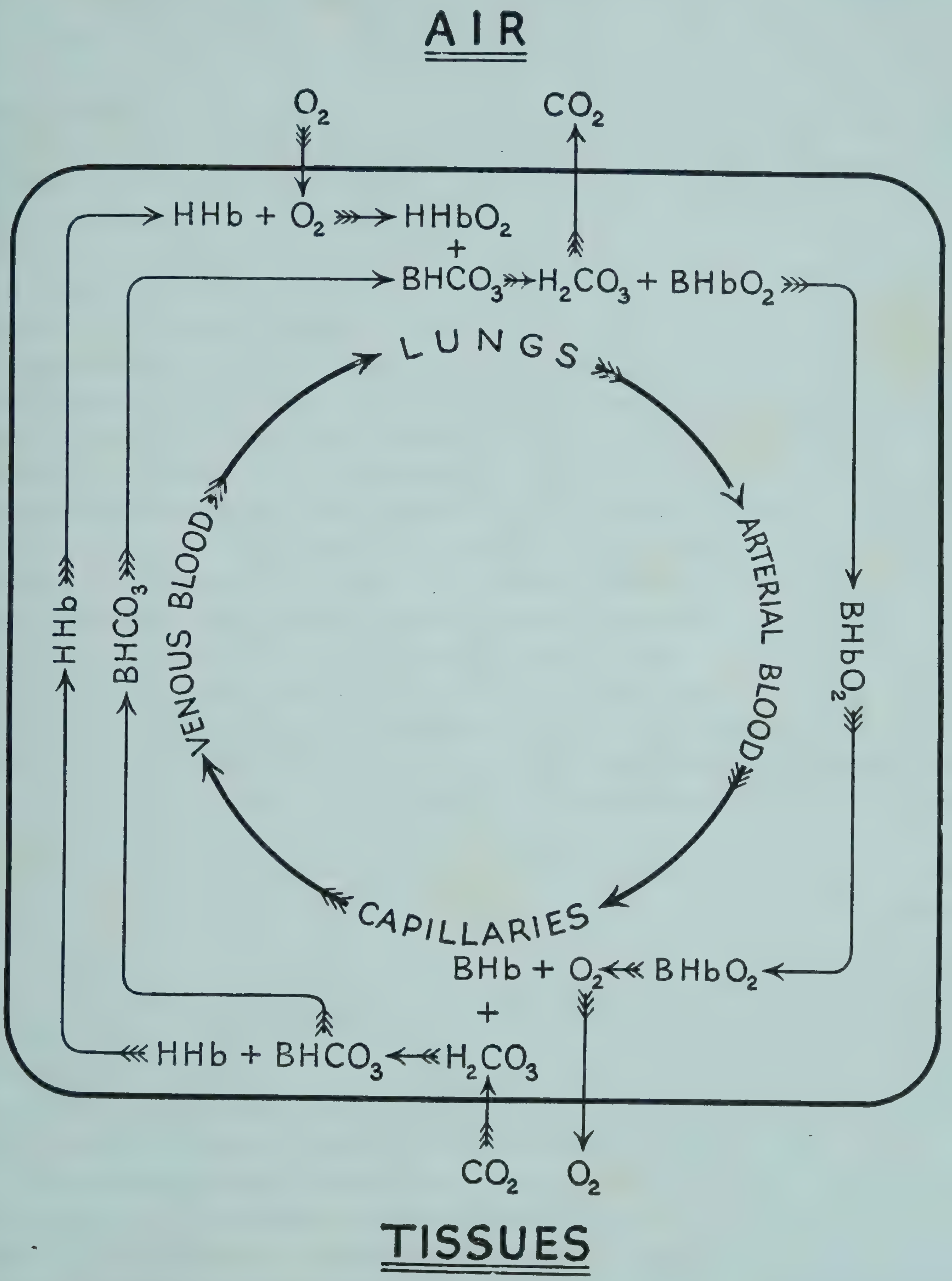

AIR
O₂
CO₂
HHb + O₂ → HHbO₂
+
BHCO₃ → H₂CO₃ + BHbO₂
LUNGS
VENOUS BLOOD
ARTERIAL BLOOD
HHb
BHCO₃
BHbO₂
CAPILLARIES
BHb + O₂ ← BHbO₂
+
HHb + BHCO₃ ← H₂CO₃
CO₂
O₂
TISSUES

FIG. 178. THE ROLE OF OXYGEN IN THE TRANSPORT OF CO_2 BY THE BLOOD.

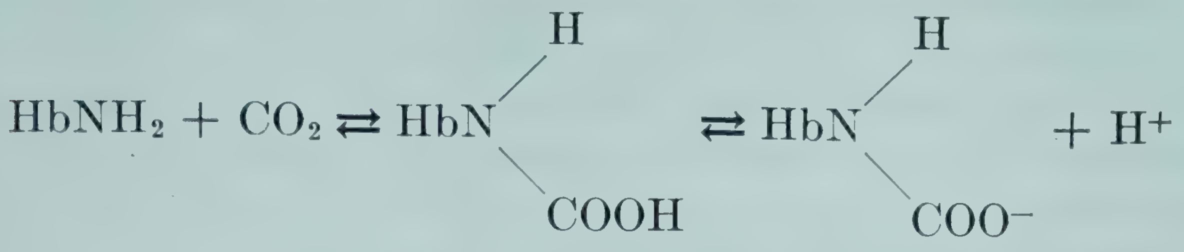
the blood is relatively small, most of the oxygen present being in the combined form of oxyhemoglobin. An equilibrium exists between the free oxygen in solution, the hemoglobin which is not combined with oxygen (so-called reduced hemoglobin), and oxyhemoglobin; this relationship can be written as follows:

$$\underset{\textbf{Reduced hemoglobin}}{Hb} + \underset{\textbf{Oxygen}}{O_2} \rightleftarrows \underset{\textbf{Oxyhemoglobin}}{HbO_2}$$

From this it can be seen that when the amount of free oxygen in the blood is lowered, as by diffusion into the tissues, the content of oxyhemoglobin will decrease and that of reduced hemoglobin will increase. This occurs when arterial blood is changed into venous blood. When venous blood reaches the lungs, it is distributed in the many capillaries of the alveoli, exposing a great surface to contact with the alveolar air. The alveolar air is separated from the blood in the pulmonary capillaries by membranes at most 2 μ in thickness; this, in addition to the fact that erythrocytes with a surface area of some 80 square meters pass through the lungs each second, accounts for the rapid and efficient uptake of oxygen. The oxygen tension of alveolar air (100 mm. Hg) is high enough to cause a considerable increase in the amount of dissolved oxygen in the blood. This in turn increases the amount of oxyhemoglobin at the expense of reduced hemoglobin, and when the aerated blood leaves the lungs as arterial blood practically all of the hemoglobin is normally in the form of oxyhemoglobin. This relation between oxygen tension and the degree of oxygenation of hemoglobin has been discussed in Chapter 22.

Now in addition to their relation to oxygen transport, the proteins oxyhemoglobin and reduced hemoglobin act as typical weak acids, being present in the blood partly in the unneutralized or free acid form (HHb, $HHbO_2$) and partly as salt ions (Hb^-, HbO_2^-). At the pH of the blood, the reduced hemoglobin ion Hb^- has a smaller base-binding capacity than has the oxyhemoglobin ion HbO_2^-, i.e., reduced hemoglobin behaves as a weaker acid than oxyhemoglobin. When HbO_2^- is converted into Hb^- in the tissue capillaries by the loss of oxygen from the blood, the concomitant release of the extra base by the reduced hemoglobin would tend to make it slightly more alkaline, if it were not for the fact that carbonic acid is entering the blood at the same time. It has been shown that, under normal circumstances, about 50 per cent of the entering carbonic acid is equivalent to this potential increase in alkalinity of the blood, i.e., will be "neutralized" without any net pH change in the blood at all. Since the carriage of this portion of the entering carbonic acid does not involve a change in pH of the blood, it is known as the *isohydric* carriage. Naturally, the exact reverse of the above-described process occurs in the lung capillaries, where reduced hemoglobin is converted by oxygenation to oxyhemoglobin. This conversion increases the base-binding capacity of the hemoglobin, withdrawing the base from $BHCO_3$ to form H_2CO_3, which is then decomposed to CO_2 and H_2O; the CO_2 diffuses out of the blood into the alveolar air of the lungs.

It may be noted here that, in addition to the carriage of a portion of the entering CO_2 by the isohydric reaction, hemoglobin enters into carbon dioxide transport in at least two other recognized ways. One of these is to act as a typical buffer, in the manner described in detail below. Another is due to the fact that hemoglobin, as a protein, is capable of combining directly with carbon dioxide to form a *carbamate*, the reaction involving the free amino groups of the hemoglobin molecule:



$$\mathrm{HbNH_2 + CO_2 \rightleftarrows HbN\begin{matrix}H\\ COOH\end{matrix} \rightleftarrows HbN\begin{matrix}H\\ COO^-\end{matrix} + H^+}$$

The formation of carbamate in this manner is a general property of proteins and amino acids and is not specific for hemoglobin, but, since hemoglobin is by far the most abundant protein in blood, a major portion of the CO_2 which is carried as carbamate is due to hemoglobin.

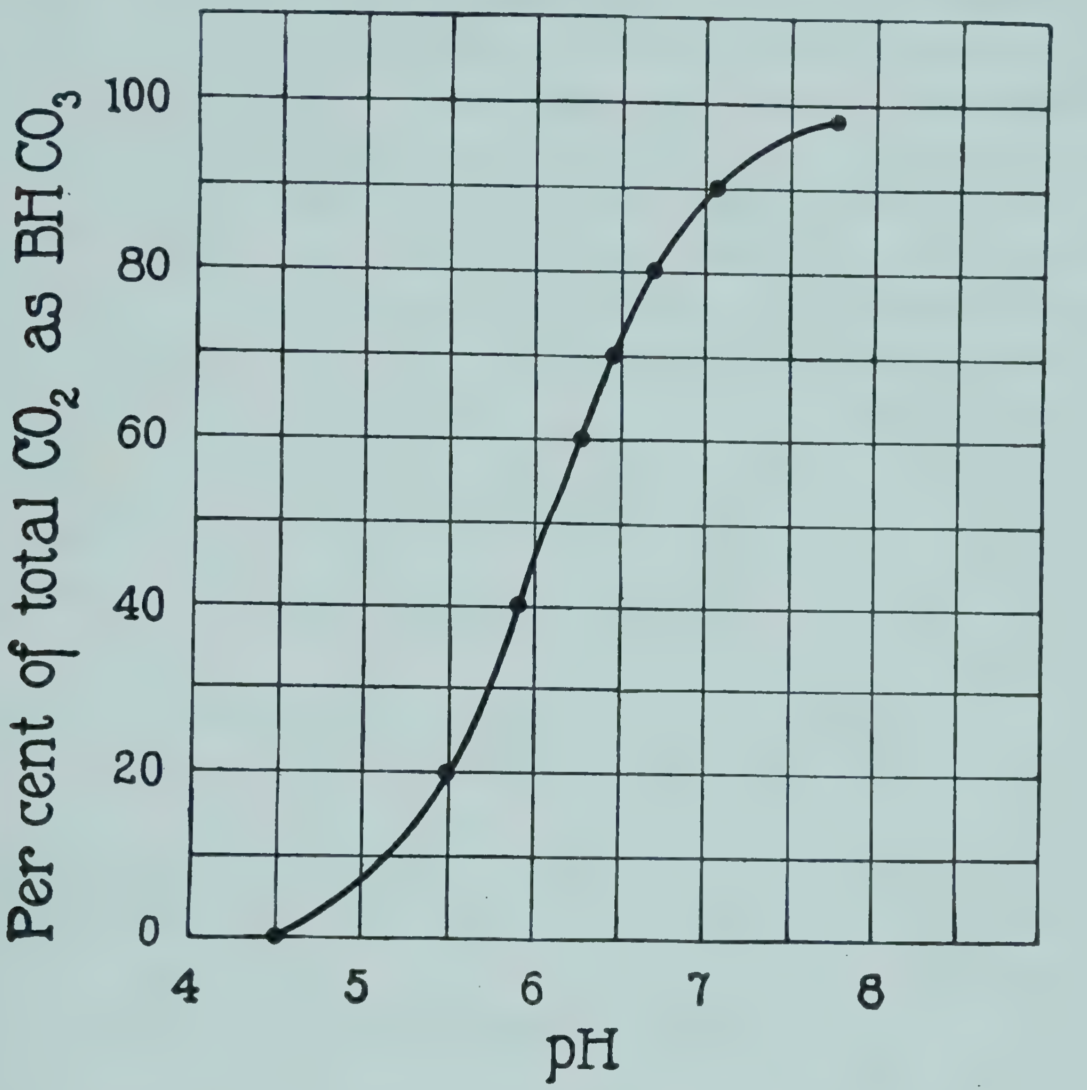


FIG. 179. TITRATION CURVE FOR THE H_2CO_3:$BHCO_3$ SYSTEM.
From Van Slyke: *Endocrinology and Metabolism,* Vol. 4, New York, Appleton, 1924.

The existence of such a direct combination of hemoglobin and CO_2 was at one time disputed, but the work of Henriques, of Roughton, and of Stadie and O'Brien has established the significance of carbamate-bound CO_2 in respiratory gas exchange. While the actual amount of carbamate-bound CO_2 in the blood is small relative to the amount of bicarbonate (see Table on p. 687), the ease of formation and breakdown of this type of compound in response to changes in CO_2 tension is such that from 20 to 30 per cent of the *extra* CO_2 added to the blood in the tissues and released in the lungs may be transported in this manner.

Role of Buffers. As has been indicated, the isohydric reaction accounts for only part of the CO_2-carrying power of the blood. When more CO_2

enters than can be neutralized in this manner, the blood becomes more acid, i.e., its hydrogen-ion concentration increases.

To understand the nature of this change, it is necessary to consider the general properties of buffer solutions (see also Chapter 1). A buffer solution ordinarily contains a weak acid and its salt, or a weak base and its salt. Such solutions are capable of taking up limited amounts of acid or base with much less change in hydrogen-ion concentration than would result from the same addition of acid or base to water or to sodium chloride solution, neither of which has buffer power. A buffer system can act therefore as a reservoir of alkali for the neutralization of the acid end products of metabolism. The efficiency of a buffer system in resisting change in reaction is greatest at the half-neutralization point, i.e., when the molar concentrations of salt and acid are equal. Fig. 179 represents a titration curve for a weak acid (carbonic), that is, a curve in which the per cent of total acid neutralized is plotted against the pH at each step in the titration. The *S*-shaped curve is characteristic of buffer acids or alkalies, and demonstrates that when 50 per cent of the acid is neutralized (ratio of salt to acid = 1), the slope (rate of change of pH) is least.

The relation between the reaction (pH) and the ratio of buffer acid (HA) to buffer salt (BA) is derived as follows. The equation for the electrolytic dissociation of a weak acid into hydrogen ions and anions is $HA \rightleftarrows H^+ + A^-$. From the law of mass action we know that the velocity of this reversible reaction in either direction is proportional to the concentrations of the reacting constituents. That is,

$$\text{Velocity (left to right)} = k_1\text{HA}$$
$$\text{Velocity (right to left)} = k_2(\text{H}^+ \times \text{A}^-)$$

in which the symbols also represent concentrations. At equilibrium the rate of reaction in each direction is the same, the equilibrium being dynamic. Therefore

$$k_1\text{HA} = k_2(\text{H}^+ \times \text{A}^-)$$

or transposing,

$$\frac{k_1}{k_2} = \frac{(\text{H}^+ \times \text{A}^-)}{\text{HA}} = K$$

K being the *dissociation constant* of the acid. Transposing again,

$$\text{H}^+ = K \times \frac{\text{HA}}{\text{A}^-}$$

In a solution of a weak acid and its salt, only a very small fraction of the anion, A^-, originates from the dissociation of the free acid, the rest coming from the dissociation of the salt, BA, into B^+ and A^-. Most salts in the concentration found in body fluids are ionized to the extent of 60 to 90 per cent. If the degree of dissociation is represented by λ, the concentration of anions $A^- = \lambda BA$ and

$$\text{H}^+ = K \times \frac{\text{HA}}{\lambda\text{BA}}$$

Since λ varies to a relatively slight extent over ranges of concentration within such limits as are found in blood constituents, it may be stated as an approximation that

$$\frac{K}{\lambda} = K_1$$

and

$$H^+ = K_1 \frac{HA}{BA}$$

K_1 is called the "apparent dissociation constant."

By definition pH = − log H^+ (see p. 30). (For example, the hydrogen-ion concentration $[H^+]$ at neutrality is 0.000,000,1 normal = 10^{-7}. Therefore the pH = $-\log 10^{-7} = -(-7) = 7$.) Taking the logarithms of both sides of the last equation,

$$\log H^+ = \log K_1 + \log \frac{HA}{BA}$$

and, changing signs, $-\log H^+ = -\log K_1 - \log \dfrac{HA}{BA}$

Substituting pH for $-\log H^+$, and, for an analogous reason, pK_1 for $-\log K_1$,

$$pH = pK_1 + \log \frac{BA}{HA}$$

This equation is known as the Henderson-Hasselbalch equation and is a most fundamental concept in the understanding of acid-base equilibrium in the body. The value of the constant pK_1 for various acids is numerically equal to the pH when the ratio of salt BA to acid HA is unity, since the log 1 = 0. Under this condition, it will be recalled, the maximum efficiency of the buffer action obtains. The following table gives the pK_1 values for the more important buffers in blood.

Buffer System	pK_1
$BHbO_2 : HHbO_2$	7.16
$BHb : HHb$	7.3
$BHCO_3 : H_2CO_3$	6.1
$B_2HPO_4 : BH_2PO_4$	6.8

With the Henderson-Hasselbalch equation in mind, consider what happens when acid is added to a buffer system. The added acid reacts with the buffer salt present to produce an equivalent increase in the amount of buffer acid, at the expense of the buffer salt, together with the salt of the entering acid which plays little, if any part in pH change and may be disregarded. The decrease in buffer salt concentration and corresponding increase in buffer acid concentration, however, must necessarily change the pH of the solution in accordance with the demands of the Henderson-Hasselbalch equation. If the solution were not buffered, the change in pH on the addition of acid would correspond to the actual amount of added H^+; in a buffered solution the change in pH is numerically much smaller, being equal to the change in the value of the logarithm of the ratio of buffer salt to buffer acid. It is for this reason that buffer solutions behave as they do in resisting gross changes in pH on the addition to the solution of acid or alkali, for it can be readily shown that an exactly analogous mechanism functions in the case of added alkali.

In a solution containing a number of different buffers, as is the case with the blood and tissues, the entering acid or alkali is buffered by all the buffer systems present, in proportion to their relative effectiveness at the given pH. Thus in the buffer systems of the blood:

$$\frac{\mathrm{BHCO_3}}{\mathrm{H_2CO_3}} \qquad \frac{\mathrm{BHb}}{\mathrm{HHb}} \qquad \frac{\mathrm{BHbO_2}}{\mathrm{HHbO_2}} \qquad \frac{\mathrm{B_2HPO_4}}{\mathrm{BH_2PO_4}} \qquad \frac{\mathrm{B\ Protein}}{\mathrm{H\ Protein}}$$

the entrance of acid will cause a decrease in concentration of all the buffer salts (numerators) and an increase in concentration of all the buffer acids (denominators), accompanied by an equivalent change in pH as required by the Henderson-Hasselbalch equation.

It is well to note here that physiological limitations influence considerably the relative significance of the various buffers in the blood. Thus the chief buffer for *carbonic acid* is hemoglobin, since carbonic acid is formed from CO_2 and H_2O only in the red cells where most of the buffering reactions for carbonic acid take place (see below). On the other hand, the major buffering action for *all acids other than carbonic acid* is exerted by the plasma bicarbonate, protein, and phosphate buffer systems.

It is significant that the pH of maximum efficiency of these buffers is below the normal pH for blood, since as the blood pH falls, change of reaction is opposed with an efficiency which increases as the danger point is approached. While the ratio $BHCO_3:H_2CO_3$ at the pH of blood is about 20:1 and hence considerably removed from the ratio of maximum efficiency, the chief significance of this system rests in the fact that H_2CO_3 promptly dissociates yielding CO_2 which is expired; thus the actual efficiency of the CO_2 system in neutrality regulation is greater than would appear to be the case from a consideration of the buffer theory alone.

Role of the Red Cells. Serum separated from the red corpuscles ("separated serum") has only a slight buffer effect and CO_2-carrying power as compared with serum in contact with the cells ("true serum"). Investigation has shown that this is because practically all of the reactions associated with the transport of CO_2 by the blood take place primarily *within the red cell itself*, the serum (or plasma) being involved in a secondary manner only. The role of the red cell and of the plasma in the uptake of CO_2 by the blood is summarized in the accompanying diagram (Fig. 180).

As indicated in the diagram, CO_2 produced metabolically in the tissues diffuses as such into the plasma. Here a small amount remains in physical solution, and some possibly reacts also with the plasma proteins ($PrNH_2$) to form carbamate, the extra equivalent of acid thus formed being buffered by the plasma buffers (A^-) in the usual way. By far the greater portion of the entering CO_2 (upward of 90 per cent) does not remain in the plasma, however, but diffuses rapidly through the red cell wall into the red cell itself.

Here it comes under the influence of the enzyme *carbonic anhydrase.* This enzyme is present in abundance in the red cell (and in certain other specialized tissues, such as the pancreas and the gastric mucosa) but is absent from the blood plasma. Its function is to act as a catalyst in the reaction $CO_2 + H_2O \rightleftarrows H_2CO_3$. By virtue of its presence within the red cell, much of the entering CO_2 is converted into H_2CO_3, which immediately dissociates to give H^+ and HCO_3^- ions. A portion of the entering CO_2 (about 20 per cent) escapes hydration, however, reacting rapidly with the hemoglobin present to form carbamate. The net effect of these two reactions, therefore, is the production within the cell of an excess of H^+ and HCO_3^- ions, together with a small amount of carbamate ions. The amounts of these ions thus produced are such as to greatly alter the normal pH and osmotic equilibrium of the cell if they were not removed in some manner.

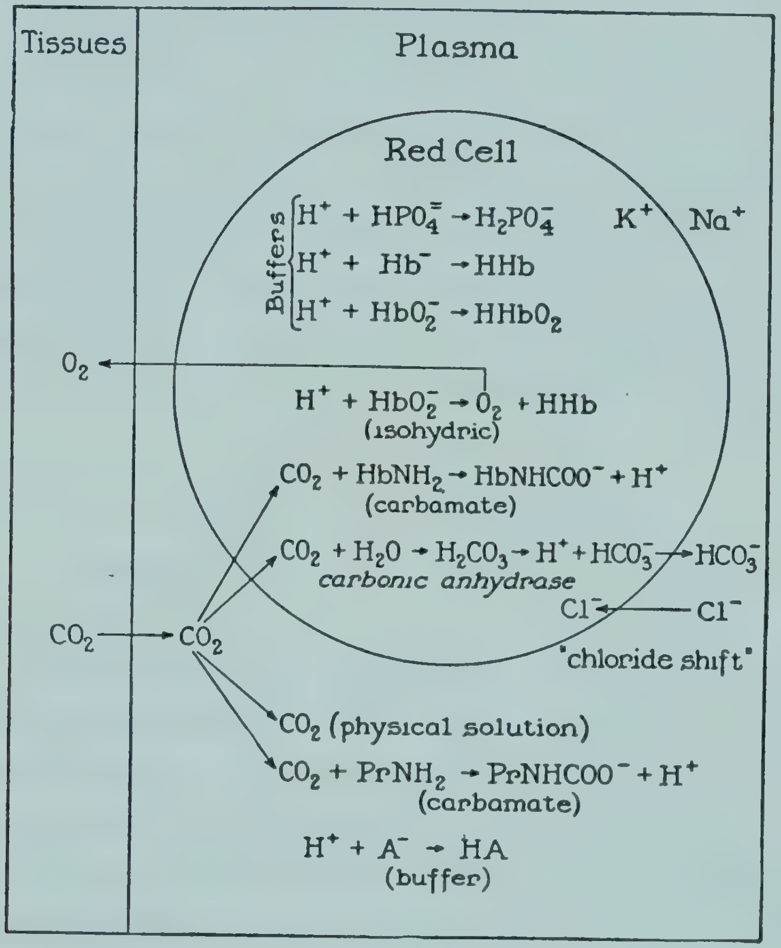

Tissues
Plasma
Red Cell
Buffers
H⁺ + HPO₄⁼ → H₂PO₄⁻
H⁺ + Hb⁻ → HHb
H⁺ + HbO₂⁻ → HHbO₂
K⁺
Na⁺
O₂
H⁺ + HbO₂⁻ → O₂ + HHb
(isohydric)
CO₂ + HbNH₂ → HbNHCOO⁻ + H⁺
(carbamate)
CO₂ + H₂O → H₂CO₃ → H⁺ + HCO₃⁻ → HCO₃⁻
carbonic anhydrase
Cl⁻
Cl⁻
"chloride shift"
CO₂
CO₂
CO₂ (physical solution)
CO₂ + PrNH₂ → PrNHCOO⁻ + H⁺
(carbamate)
H⁺ + A⁻ → HA
(buffer)

FIG. 180. PROCESSES INVOLVED IN THE UPTAKE OF CARBON DIOXIDE BY BLOOD.

The H^+ ions are removed in two ways, by the isohydric reaction and by buffer action. It has already been pointed out that oxyhemoglobin (HbO_2) is a stronger acid than reduced hemoglobin (Hb) at the pH of the blood. This means in effect that hemoglobin which has lost its oxygen has a lesser base-binding capacity at a given pH than has oxyhemoglobin. Thus the liberation of oxygen from the blood to the tissues creates a situation which brings about the release of base from the hemoglobin without involving any pH change at all. This process, under the conditions prevailing in normal blood, accounts for the disposal of over 50 per cent of the extra H^+ ions produced within the red cell from the entering CO_2. The remaining H^+ ions react with the various buffer salt ions present in the cell to form an equivalent amount of un-ionized buffer acids. Since this typical buffer reaction involves a change in the ratio of buffer salt to buffer acid, the pH of the cell contents will change as demanded by the Henderson-Hasselbalch equation, and it is this which accounts for the slight change in pH which follows the uptake of CO_2 by the cells.

The fate of the extra HCO_3^- ions must now be considered. If they remained within the cell, the increased osmotic pressure would cause the cell to take up water and swell to a size far beyond physiological limits. It has been found by chemical analysis of cells and plasma that most of the extra HCO_3^- ions diffuse out of the cell into the plasma, being replaced by an equivalent amount of Cl^- ions which diffuse from the plasma into the cell. This process is called the *chloride shift* and it has been estimated that as much as 80 per cent or more of the *extra* bicarbonate of venous plasma (as compared to arterial plasma) is due to diffusion from the red cells.

The chloride shift has been attributed by Van Slyke to the existence of a Donnan equilibrium (see p. 12) across the red cell membrane. It can be shown that, in the case of the red cell, it is a consequence of the Donnan theory that at osmotic equilibrium the ratio of bicarbonate-ion concentrations in cell and plasma is proportional to the ratio of chloride-ion concentrations in cell and plasma, i.e.,

$$\frac{[HCO_3^-]_{cells}}{[HCO_3^-]_{plasma}} = k \cdot \frac{[Cl^-]_{cells}}{[Cl^-]_{plasma}}$$

An increase of bicarbonate-ion concentration within the cell disturbs this equilibrium and it is partially restored by migration of bicarbonate ions from the cell to the plasma, accompanied by an equivalent migration of chloride ions from the plasma to the cell.[1] At the same time, a certain amount of water diffuses from the plasma into the red cell, and this accounts for the slight increase in size of the cells of venous blood as compared to arterial blood.

The phenomena that have just been described take place during the uptake of CO_2 by the blood in the tissue capillaries to form venous blood. When the venous blood reaches the lungs, the entrance of oxygen reverses all of these processes. Bicarbonate in the cell is converted into carbonic acid which is dehydrated to CO_2 and diffuses out of the cells into the plasma, from whence it passes into the alveolar air of the lungs. Bicarbonate ions migrate from the plasma to the cell and undergo the same reactions, chloride ions returning from the cell to the plasma at the same time. The various other reactions are likewise reversed in an analogous manner. Thus the red cell is carried through the entire mechanism first in one direction and then in the other direction as it makes a complete circuit of the body.

The quantitative nature of the changes that normally take place in the blood in its transition from the arterial to the venous state is illustrated by the data of the following table, adapted from Stadie and O'Brien.[2]

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[1] The hemoglobin ions likewise enter into this equilibrium. For a detailed discussion of the Donnan equilibrium as it is applied to the chloride shift, the reader is referred to Peters and Van Slyke: *Quantitative Clinical Chemistry*, Vol. I; see also Hitchcock: *Physical Chemistry for Students of Biology and Medicine*, 3d ed.

	Arterial Blood			*Venous Blood*			*Difference*			
	Plasma	*Cells*	*Whole Blood*	*Plasma*	*Cells*	*Whole Blood*	*Plasma*	*Cells*	*Whole Blood*	
										Per cent
Hematocrit	0.600	0.400	..	0.596	0.404	..	..	..	..	..
O_2 saturation, per cent	..	96.0	..	..	74.0	..	..	..	..	..
pH	7.45	7.12	..	7.43	7.11	..	..	..	..	..
pCO_2, mm. Hg	40.0	40.0	40.0	45.4	45.4	45.4	..	..	..	..
Free CO_2, vol. per cent	1.6	0.8	2.4	1.8	0.9	2.7	0.2	0.1	0.3	8.0
Bound CO_2, vol. per cent	34.1	11.8	45.9	36.3	13.1	49.4	2.2	1.3	3.5	92.0
Bicarbonate, vol. per cent	33.1	9.8	42.9	35.2	10.5	45.7	2.1	0.7	2.8	74.0
Carbamate, vol. per cent	1.0	2.0	3.0	1.1	2.6	3.7	0.1	0.6	0.7	18.0
Total CO_2, vol. per cent	35.7	12.6	48.3	38.1	14.0	52.1	2.4	1.4	3.8	100.0

Study of this table shows the following:

1. In its passage through the tissues of the body, the arterial blood of the subject being studied has picked up about 3.8 ml. of CO_2 per 100 ml. of whole blood. This is an increase of about 8 per cent in the total CO_2 content of the blood. With the R.Q. assumed to be 0.85, a normal value, this corresponds to the liberation of about 4.5 ml. of O_2 from the blood at the same time, which is responsible for the change in oxygen saturation from 96 per cent to 74 per cent.

2. The increased CO_2 content of venous blood is accompanied by a rise in CO_2 tension of about 5 mm. of Hg, and a fall in pH of about 0.01 to 0.02 pH units.

3. Of the total CO_2 of blood, almost 90 per cent is in the form of bicarbonate, of which about three-fourths is found in the plasma and one-fourth in the cells. The remaining 10 per cent of the total CO_2 is about equally distributed between the forms of free CO_2 and carbamate. Plasma contains about twice as much free CO_2 and about half as much carbamate-bound CO_2 as is found in the cells.

4. Of the 3.8 volumes per cent of CO_2 added to the blood in the change from arterial to venous blood, which are ultimately released in the lungs, about three-fourths appears as extra bicarbonate, about one-fifth as carbamate, and the remainder as an increase in the free CO_2 content. The extra free CO_2 and extra bicarbonate are both distributed between cells and plasma in about the same proportion as that already present; almost all of the extra carbamate is found in the cells.

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[2] *J. Biol. Chem.*, **117**, 439 (1937).

5. Most of the extra bicarbonate of venous blood is found in the *plasma*. Now it has been shown that the passage of blood through the capillaries is far too rapid to permit any appreciable hydration of CO_2 to H_2CO_3 (a necessary preliminary to the formation of bicarbonate) without the intervention of carbonic anhydrase, and this enzyme is found only in the cells. It follows, therefore, that most of the *extra* bicarbonate of venous plasma must have come from the cells. This is accomplished by virtue of the "chloride shift" mechanism, an equivalent amount of chloride leaving the plasma and entering the cells.

Relative Importance of the Various CO_2-Carriers of the Blood. A CO_2-carrier of the blood has been broadly defined by Van Slyke as any substance present in the blood which increases the amount of CO_2 that may be taken up by arterial blood without a change in pH beyond the normal difference between venous and arterial blood. From this definition it is clear that the major CO_2-carriers of the blood include hemoglobin, bicarbonate, the plasma proteins, and phosphates. It is also clear that by far the most important CO_2-carrier of normal blood is hemoglobin, since 80 per cent or more of the CO_2 carriage by the blood is mediated directly or indirectly through the presence of hemoglobin. Thus this compound plays as important a part in neutrality regulation and the transport of CO_2 as it plays in its more obvious function in O_2 transport.

Role of the Lungs. Due to the ease of elimination of CO_2 by respiration, the regulation of the neutrality of the blood is largely controlled by the $H_2CO_3:BHCO_3$ system. From 20 to 40 liters of N/1 acid as CO_2 is lost each day by way of the lungs. By means of variation in the rate and depth of respiration and the rate of blood flow through the lungs, opportunity is afforded for very delicate adjustment of the hydrogen-ion concentration. The nervous control of the respiratory mechanism resides in the respiratory center in the medulla. Increases in CO_2 tension or hydrogen-ion concentration call forth corresponding responses in the ventilation of the lungs, and since changes in either factor take place concurrently, it is difficult to distinguish the effects of one from the other. It is probable that an increase in hydrogen ions in the respiratory center itself, secondary to that of the blood, may be the activating factor. Such increases may come about through diffusion of CO_2 or H^+ from the blood or by acid production within the center. The latter may explain the greater respiratory response which results from an insufficient oxygen supply to the center.

The rate of elimination of waste products through the lungs and the kidneys is partly controlled by the rate of blood flow through those organs. The diminished blood pressure following excessive pulmonary ventilation has been shown to be due to the loss of CO_2 rather than to the resultant rise in pH *per se*. There is, however, no constant relation between blood pressure and CO_2 tension among different individuals.

Role of the Kidneys. The role of the kidneys in neutrality regulation is concerned chiefly with the conservation of "fixed base" (sodium, potassium) to the organism. If a strong acid produced by metabolism within the tissues is neutralized by reaction with bicarbonate or basic phosphate to produce a salt, the base component of the salt thus formed (e.g., the Na of NaCl) is no longer a direct part of the alkali reserve of the body, and if present in excess will be excreted in the urine. If, however, the alkali reserve is low so that the organism needs to conserve fixed base, as when acid production exceeds the supply of available base, the kidney has the ability to excrete an acid urine (down to a pH as low as 4.8) as one means of conserving base. According to Pitts, this is probably accomplished by the reabsorption of base ions from the glomerular ultrafiltrate and their replacement by hydrogen ions secreted into the tubular lumen. In effect, the following reaction occurs:

$$\underset{\textbf{(from blood)}}{Na_2HPO_4} + \underset{\textbf{(from kidney)}}{H^+} \rightarrow \underset{\textbf{(to urine)}}{NaH_2PO_4} + \underset{\textbf{(to blood)}}{Na^+}$$

The increase in the relative amount of acid phosphate to basic phosphate in the urine accounts for the more acid pH of the urine. If the urine is titrated with alkali back to the pH of the blood (see "Titratable Acidity of Urine" in Chapter 31), a measure of the extent to which the above reaction has contributed to base conservation will be obtained.

A second and equally important mechanism whereby the kidneys conserve fixed base is by the synthesis of ammonia, probably from glutamine and amino acids.[3] The ammonia is excreted in preference to fixed base, as follows:

$$\underset{\textbf{(from blood)}}{NaCl} + \underset{\textbf{(from kidney)}}{NH_4^+} \rightarrow \underset{\textbf{(to urine)}}{NH_4Cl} + \underset{\textbf{(to blood)}}{Na^+}$$

The extent of ammonia excretion is related to the need for base conservation, ordinarily being low or zero in alkalosis and high in acidosis. Thus by determining both the ammonia excretion and the titratable acidity of the urine, the extent of acid excretion by the body may be evaluated, and hence knowledge be gained concerning the state of acid-base balance.

It sometimes happens that there is an excess of base requiring excretion, as on diets high in alkaline ash or after administration of bicarbonate. In this event, extra base and bicarbonate will be found in the urine and the pH may rise to as high as 8.0.

The pH and H_2CO_3:$BHCO_3$ Ratio. Inasmuch as the chief factor in the regulation of blood reaction is the H_2CO_3:$BHCO_3$ system, the relationship of variations in the latter to pH should yield valuable information concerning changes in the acid-base balance of the body. Applying the equation for the dissociation of a weak acid (see p. 682) to this case, the so-called Henderson-Hasselbalch equation is obtained.

$$\mathrm{pH} = \mathrm{p}K_1 + \log \frac{\mathrm{BHCO_3}}{\mathrm{H_2CO_3}}$$

The value of the constant $\mathrm{p}K_1$ for blood plasma is 6.10. By measuring any two of the remaining variables, the third is of course determined. If we plot changes in bicarbonate concentration [$BHCO_3$] as ordinates, against carbonic acid [H_2CO_3] as abscissas, a straight line will result for any given ratio of the two, and hence for any given pH. The slope of this line will increase or decrease with corresponding changes in the [$BHCO_3$]:[H_2CO_3] ratio. The maximal as well as the normal ranges of these factors are depicted in Fig. 181, constructed by Van Slyke. Since it was desired to use the customary form of CO_2 absorption curves, the coordinates are expressed in terms of total CO_2 values, [$BHCO_3 + H_2CO_3$], as ordinates, and CO_2 tensions as abscissas. It will be noted that nine areas are set off by the three levels (high, normal, or low) of each of the two variables. The nine areas represent conditions of acid or alkali excess or deficit, which are either *compensated* (pH normal) or *uncompensated* (pH above or below normal). That is, a condition of alkali deficit (low alkali reserve) may be either compensated by a corresponding diminution in carbonic acid so that the [$BHCO_3$]:[H_2CO_3] ratio remains normal (about 20:1), or it may be uncompensated by the failure to remove sufficient CO_2, in which case the pH falls and the condition of uncompensated alkali deficit results. It is therefore obvious that acidosis cannot be regarded as merely a lowering of the pH, since the acidosis may accompany a deficit of CO_2 so marked as to result in a more alkaline reaction of the blood. Moreover, an abnormally acid reaction may occur even with an increase of alkali reserve provided CO_2 is present to excess. In order, therefore, to combine the effects of both alkali reserve and reaction, Van Slyke has broadly defined acidosis as a condition caused "by the formation or absorption of acids at a rate exceeding that of their elimination . . . [which] . . . may be considered to have caused an abnormal state when it has either

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[3] Van Slyke, Phillips, Hamilton, Archibald, Futcher, and Hiller: *J. Biol. Chem.*, **150**, 481 (1943).

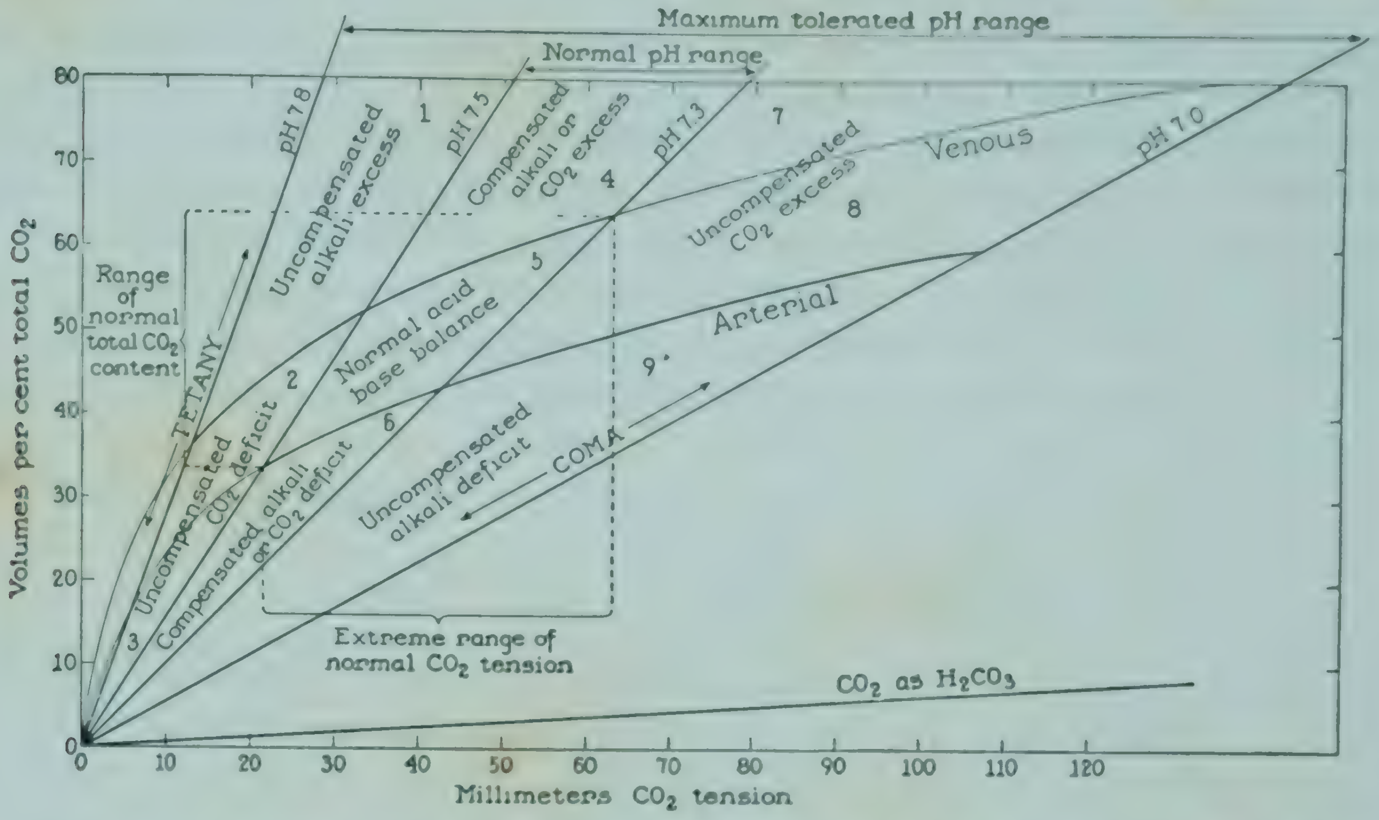

FIG. 181. NORMAL AND ABNORMAL VARIATIONS OF THE $BHCO_3$, H_2CO_3, CO_2 TENSION, AND pH IN OXYGENATED HUMAN WHOLE BLOOD DRAWN FROM RESTING SUBJECTS AT SEA LEVEL. (VAN SLYKE.)

The bicarbonate CO_2 at any point is obtained by subtracting from the total CO_2 the relatively small amount present as H_2CO_3 indicated by the slanting line near the bottom of the illustration.

DISTURBANCES OF ACID-BASE EQUILBRIUM OF BLOOD

Area (Fig. 181)	*Acid-base Balance*	*Conditions*	*Associated Symptoms*	*Compensatory Mechanisms*
1 Uncompensated Alkali Excess	$[BHCO_3]$ increased without proportionate rise in $[H_2CO_3]$, therefore pH increased	Overdosage of $NaHCO_3$. Excessive vomiting (pyloric obstruction) or gastric lavage (loss of HCl). X-ray or radium treatment	If marked, tetany	Diminished respiration (rise in alveolar CO_2) to hold back CO_2 Diuresis and increased $NaHCO_3$ excretion
2–3 Uncompensated CO_2 Deficit	$[H_2CO_3]$ decreased without proportionate fall in $[BHCO_3]$; therefore pH increased	Hyperpnea, voluntary or induced (oxygen want, e.g., at high altitudes) Fever Hot baths	If marked, tetany	Retention of acid metabolites (low NH_3 and titratable acidity of urine) Excretion of $NaHCO_3$
4 Compensated Alkali or CO_2 Excess	$[BHCO_3]$ (or $[H_2CO_3]$) increased but balanced by proportionate rise in $[H_2CO_3]$ (or $[BHCO_3]$); therefore pH normal	*Alkali excess* $NaHCO_3$ therapy, with slow absorption *CO_2 excess* Retarded gas exchange (e.g., emphysema) with CO_2 tension chronically increased	Cyanosis due to deficient oxygen exchange	CO_2 retention $BHCO_3$ retention
5 Normal	$[BHCO_3]$ and $[H_2CO_3]$ normal at ordinary altitudes			
6 Compensated Alkali or CO_2 Deficit	$[BHCO_3]$ (or $[H_2CO_3]$) decreased but balanced by proportionate fall in $[H_2CO_3]$ (or $[BHCO_3]$); therefore pH normal	*Alkali deficit* Accelerated production (e.g., diabetes) or retarded elimination (e.g., nephritis) of nonvolatile acids Experimental acid intoxication Diarrheal acidosis of infancy (marasmus) *CO_2 deficit* Overventilation at high altitudes (oxygen want)	Hyperpnea	Increased respiration ("blowing off CO_2") Accelerated NH_3 formation and acid excretion Same as in Areas 2 and 3
7–8 Uncompensated CO_2 Excess	$[H_2CO_3]$ increased without proportionate rise in $[BHCO_3]$; therefore pH decreased	Retarded respiration as in pneumonia (physical obstruction) or morphine narcosis (deadening of respiratory center) Experimental rebreathing Cardiac decompensation	Dyspnea	Increased respiration. Accelerated NH_3 formation and acid excretion. Probable shift of acid from blood to tissue
9 Uncompensated Alkali Deficit	$[BHCO_3]$ decreased without proportionate fall in $[H_2CO_3]$; therefore pH decreased	Terminal stages of nephritic acidosis, and diabetic acidosis (compensated by insulin therapy) Deep ether anesthesia Certain cardiac cases Eclampsia	Dyspnea	Increased respiration. Increased acid excretion and NH_3 formation (except probably in nephritis)

increased the hydrion concentration of the blood or lowered its alkali reserve below the extreme normal limits." The conditions which fall into the different areas have been observed both clinically and experimentally.

Disturbances of the Acid-base Balance. These are divided into two clinical types by Peters and Van Slyke, viz., (1) metabolic types, in which the primary disturbance is in the relation between alkali and acids other than H_2CO_3, and (2) respiratory types, in which the primary disturbance involves the CO_2 content of the blood. In the table on p. 691 the more important conditions associated with disturbance of acid-base balance are shown, together with their causes and the physiological mechanisms brought into play for their compensation.

KETOSIS. Ketosis is the condition in which abnormal amounts of β-hydroxybutyric acid, acetoacetic acid, and acetone accumulate in the body fluids and may be readily detected in the urine. These three compounds are commonly known as the *acetone bodies*. Of the three acetone bodies, β-hydroxybutyric acid and acetoacetic acid are primary products, acetone being derived from the decarboxylation of acetoacetic acid. They appear to have their origin principally from the metabolism of the fatty acids of fats, and to a lesser degree from certain amino acids resulting from protein cleavage. This is discussed in detail in Chapter 33. They are apparently formed in the liver, and then further oxidized in the other tissues of the body where they are capable of furnishing a large amount of energy. In diabetes mellitus, the body either does not possess the normal power of oxidizing these substances or else they are produced in excessive amounts. At any rate, we find them in the blood and urine in abnormal quantity. Likewise, in the absence of dietary carbohydrate and in other conditions of faulty carbohydrate metabolism, they are also increased in amount in both blood and urine. Ketosis as it appears in the human organism is not entirely the same as in other species, such as the rat, dog, rabbit, goat, pig, and cow. Rats are very resistant to the development of ketosis.

The significance of ketosis in connection with acid-base balance lies in the fact that acetoacetic acid and β-hydroxybutyric acid exist in the blood and urine largely in the form of their alkali salts. Their production has therefore required an equivalent amount of body base for neutralization. Excessive amounts produce a severe demand on available base, and when they are excreted in the urine most of this base is lost to the organism. The acidosis of diabetes mellitus may be largely due to ketosis. Acidosis occurs in many conditions, however, without a concomitant ketosis.

The presence of acetone bodies in the urine in appreciable quantity was originally taken as an index of acidosis, the severity of which was judged by the estimation of the amount of these substances present in the urine. That this is not a reliable index is shown by the occasional observation of a pronounced acidosis with no appreciable increase in urinary acetone bodies. A high urinary ammonia coefficient (ammonia N:total N) was once looked upon as an indication of acidosis. However, this factor is not specific in diagnosis in spite of the fact that the majority of such cases show a high urinary ammonia value because certain dietetic changes may produce high urinary ammonia. Fatal acidosis has been observed in uremia, and in nutritional disorders of infants, with no pronounced increase in the ammonia coefficient. In this connection, it is possible that in renal disability the ammonia-forming function of the kidneys is impaired.

Conditions of disturbed acid-base balance are best diagnosed and their course followed not by the determination of acetone bodies or ammonia in either urine or blood, but by the determination of certain other factors which are more or less typical of acidosis, such as the quantitative estimation of the alkali reserve of the blood and of its pH.

METHODS

1. *Simple Demonstration of the Presence of Carbon Dioxide in Expired Air.* Into each of two small flasks or large test tubes introduce 25 ml. of a clear saturated solution of barium hydroxide. After an ordinary inspiration, expire through a bent glass tube or pipet dipped beneath the surface of the solution in the first flask. Repeat the experiment with the second flask, but hold the breath as long as possible after the inspiration before breathing out through the tube. Note the relative amounts of precipitate of barium carbonate formed. To another flask add 30 ml. of water, 1 or 2 drops of barium hydroxide solution, and a few drops of 0.04 per cent phenol red. Expire into this solution until a change takes place. What does this change indicate?

2. *Alkali Reserve: Direct Method.*

(a) CARBON DIOXIDE CAPACITY OF THE PLASMA (VAN SLYKE AND CULLEN): PRINCIPLE. The plasma from oxalated blood is shaken in a separatory funnel filled with an air mixture whose carbon dioxide tension approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known quantity of the saturated plasma is then acidified within a suitable pipet, and its carbon dioxide is liberated by the production of a partial vacuum. The liberated carbon dioxide is then placed under atmospheric pressure, its volume carefully measured, and the volume corresponding to 100 ml. of plasma calculated.

Apparatus. The apparatus used in the estimation of the carbon dioxide content of the plasma is illustrated in Figs. 182 and 183. It is made of strong glass in order to stand the weight of mercury without danger of breaking, and is held in a strong screw clamp the jaws of which are lined with thick pads of rubber. In order to prevent accidental slipping of the apparatus from the clamp, an iron rod of 6 or 8 mm. diameter should be so arranged as to project under cock *f* between *c* and *d*.

Three hooks or rings at the levels, 1, 2, and 3 serve to hold the leveling bulb at different stages of the analysis. The bulb is connected with the bottom of the apparatus by a heavy-walled rubber tube.

It is necessary, of course, that both stopcocks should be properly greased and airtight, and it is also essential that they (especially *f*) shall be held in place so that they cannot be forced out by pressure of the mercury. Rubber bands may be used for this purpose but elastic cords of fine wire spirals, applied in the same manner as rubber bands, are stronger and more durable. Later models of this apparatus are equipped to overcome this difficulty.

After a determination has been finished, the leveling bulb is lowered without opening the upper cock, and most of the mercury is withdrawn from the pipet through *c*. The water solution from *d* is readmitted and the leveling bulb being raised to position 1, the water solution, together with a little mercury, is forced out of the apparatus through *a*.[4]

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[4] It is well to have a funnel draining into a special vessel to catch the water residues and mercury overflow from *a*. A considerable amount of mercury is thus regained if many analyses are run. It requires only straining through cloth or chamois skin to prepare it for use again.

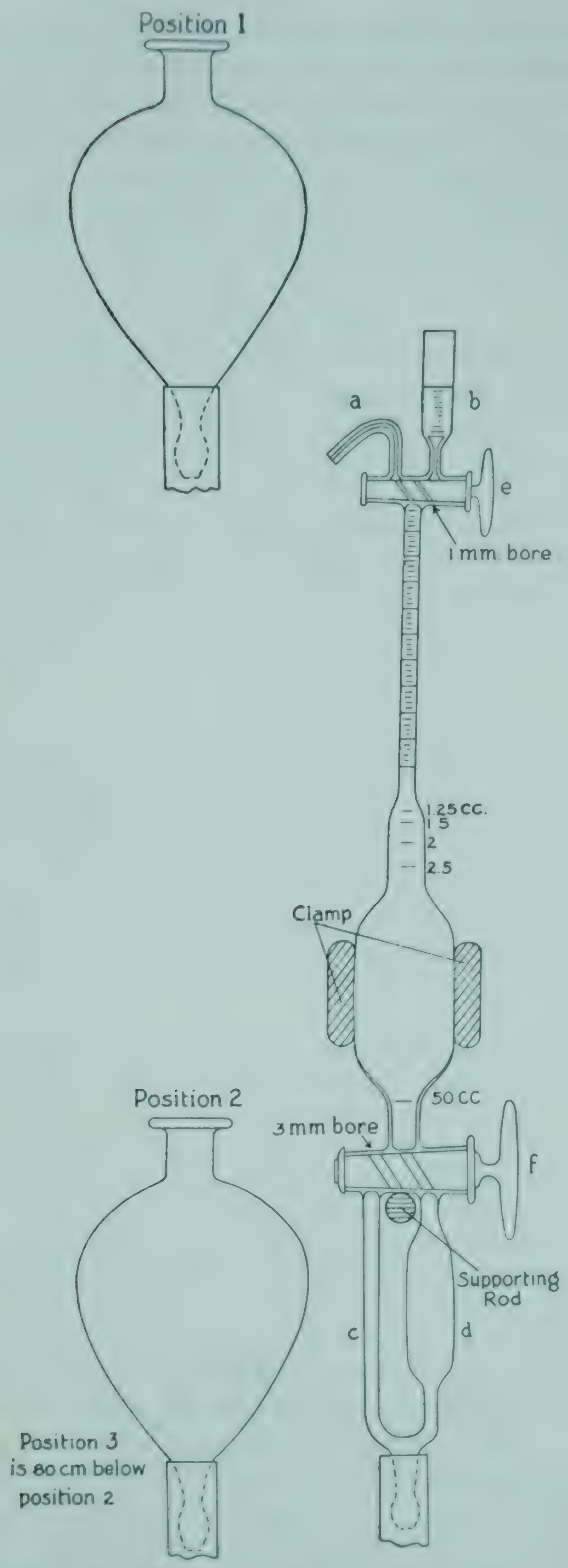
Position 1
a
b
e
1 mm. bore
1.25 cc.
1.5
2
2.5
Clamp
Position 2
50 cc
3 mm bore
f
Supporting Rod
c
d
Position 3 is 80 cm below position 2

FIG. 182. VAN SLYKE VOLUMETRIC CARBON DIOXIDE APPARATUS.

Procedure. Drawing the blood.[5] About 6 or 7 ml. of venous blood are aspirated into a centrifuge tube (see Fig. 183) containing a little powdered potassium oxalate and some paraffin oil. The tube is subjected to a minimum of agitation after the blood is in it. The slight amount of agitation necessary to assure mixture with the oxalate is accomplished by stirring with the inlet tube, rather than by inverting or shaking. The tube and contents are then centrifuged.

Saturation of Plasma with Carbon Dioxide. After centrifuging, about 3 ml. of the plasma[6] are transferred to a 300-ml. separatory funnel, arranged as in Fig. 184, and the air within the funnel is displaced by either alveolar air from the lungs of the operator or a 5.5 per cent carbon dioxide–air mixture from a tank. This latter procedure is preferred, since error due to incomplete filling of the separatory funnel with air containing carbon dioxide at the proper tension is less likely to occur. The gas must be passed through a wash bottle containing water before entering the funnel.

When alveolar air is used, the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the glass beads and separatory funnel. The stopper of the funnel should be inserted just before the expiration is finished, so that there is no opportunity for air to be drawn back into the funnel. In order to saturate the plasma the separatory funne

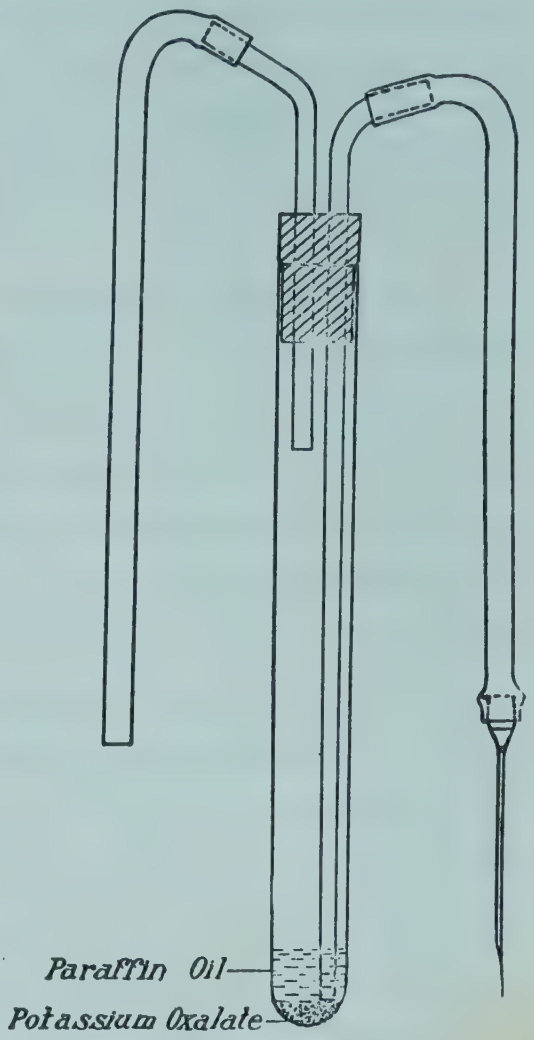

FIG. 183. ASPIRATING TUBE USED TO COLLECT BLOOD.

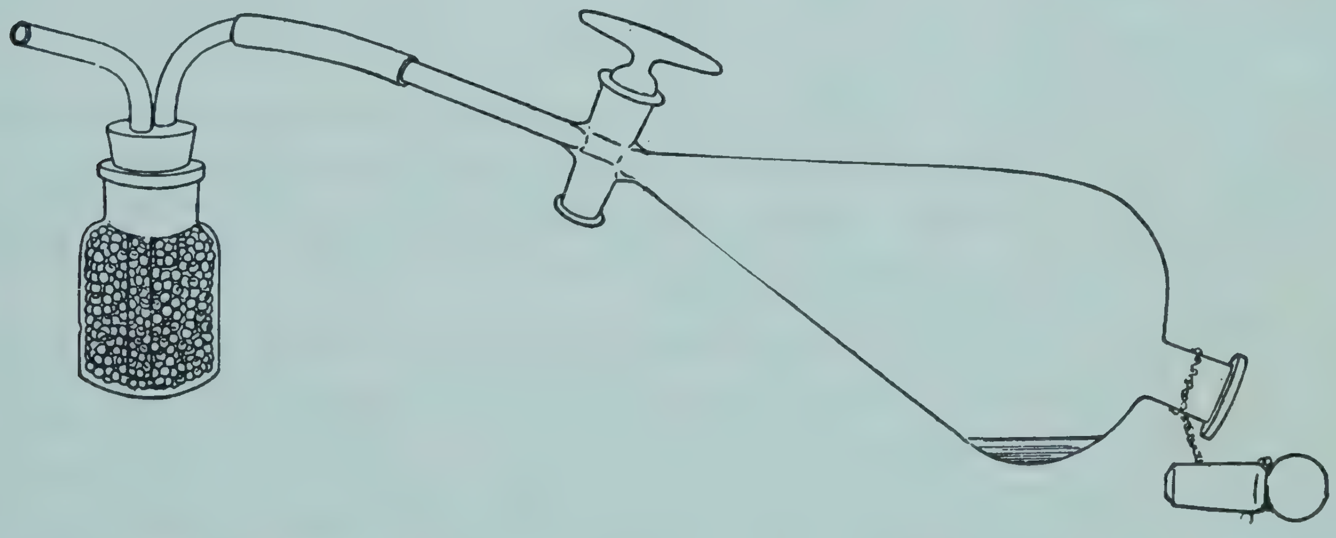

FIG. 184. SEPARATORY FUNNEL USED IN SATURATING BLOOD PLASMA WITH CARBON DIOXIDE.

Courtesy, *J. Biol. Chem.*, **30**, 289 (1917).

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[5] For at least an hour before the blood is drawn the subject should avoid vigorous muscular exertion as this, presumably because of the lactic acid formed, lowers the bicarbonate of the blood.

[6] If it is desired to keep the plasma for the estimation of carbon dioxide at a later time, it should be transferred to a paraffined tube, covered with a layer of paraffin oil, stoppered, and kept cold; under which conditions it is claimed that, if sterile, it may be kept for over a week without alteration of its carbon dioxide capacity.

is turned end over end for 2 minutes, the plasma being distributed in a thin layer as completely over the surface of the funnel's interior as is possible. After saturation is completed, the funnel is placed upright and allowed to stand for a few minutes until the fluid has drained from the walls and gathered in the contracted space at the bottom of the funnel.

Determination of Carbon Dioxide. The cup should be washed out with water and 1 ml. of distilled water run in, and together with the entire apparatus should be filled with mercury to the top of the capillary tube by placing the leveling bulb of mercury in position 1. A sample of 1 ml. (or 0.5 ml. in case the amount of plasma available is very small) accurately pipeted, is allowed to run into the cup *b* in the apparatus represented in Fig. 182, the tip of the pipet remaining below the surface of the water as it is added. Add 1 drop of caprylic alcohol.[7]

With the mercury bulb at position 2 and the cock *f* in the position shown in the illustration, the plasma, water, and alcohol are admitted from the cup into the 50-ml. chamber, leaving just enough above the cock *e* to fill the capillary so that no air is introduced when the next solution is added. In the case of whole blood, some corpuscles settle to the bottom of the cup. These are suspended in the water by stirring them up with the last 0.5 ml. of water after the first 0.5 ml. has passed into the chamber. Finally, 0.5 ml. of 5 per cent sulfuric acid is run in.[8]

It is not necessary that exactly 1 ml. of wash water and 0.5 ml. of acid shall be taken, but the total volume of the water solution introduced must extend exactly to the 2.5-ml. mark on the apparatus, if the table on p. 700 is to be used.

If the amount of plasma available is small, a little more than 0.5 ml. is saturated in a 50-ml. funnel, and exactly 0.5 ml. used for the estimation of carbon dioxide. In this case, the volume of distilled water and acid used to wash the plasma into the apparatus is halved, so that the total volume of water solution introduced is only 1.25 ml., and in the calculation the observed volume of gas is multiplied by 2.

After the acid has been added, a drop of mercury is placed in *b* and allowed to run down the capillary as far as the cock in order to seal the latter. A copper wire may be used to expel a bubble of air which may be trapped in the capillary. Whatever excess of sulfuric acid remains in the cup is washed out with a little water, using a medicine dropper.

The mercury bulb is now lowered and hung at position 3, and the mercury in the pipet is allowed to run down to the 50-ml. mark, producing a Torricellian vacuum in the apparatus. When the mercury (not the water) meniscus has fallen to the 50-ml. mark, the lower cock is closed and the pipet is removed from the clamp. Equilibrium of the carbon dioxide between the 2.5 ml. of water solution and the 47.5 ml. of free space in the apparatus is obtained either by mechanical shaking for 1 minute, or by turning the pipet upside down 15 or more times, thus thoroughly agitating the contents. The pipet is then replaced in the clamp.

After extraction of the gas the lower cock is opened, admitting the mercury into the extraction chamber rapidly until the meniscus of the water solution

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[7] It is desirable to keep the amount of caprylic alcohol small, as larger amounts may appreciably increase results. With plasma 0.02 ml. is sufficient to prevent foaming and is measured most conveniently from a buret made by fusing a capillary stopcock to a pipet graduated in 0.01-ml. divisions.

[8] With whole blood, in place of sulfuric acid use lactic acid made by diluting 1 volume of concentrated acid (specific gravity 1.20) to 10 volumes with water.

reaches the contracted upper portion of the chamber. At this moment the lower cock is partially closed and the remainder of the mercury is admitted at a rate sufficiently retarded to prevent oscillation of the water column in the calibrated portion of the apparatus when pressure equilibrium is reached. The pressure is then adjusted by placing the mercury surface in the leveling bulb above the mercury meniscus in the chamber by a height equal to one-thirteenth that of the water column, in order to balance the latter. After some practice controlled by a centimeter rule, one can estimate this level with the eye to within 2 mm. of mercury, which is sufficiently accurate for most purposes. When the pressure has been adjusted, the lower cock of the apparatus is closed. The gas volume may then be read at leisure.

When, as in most plasma analyses, the CO_2 is not reabsorbed, no washing of the apparatus is necessary before using it for another determination, since the acid solution which wets the walls of the chamber contains a negligible amount of CO_2.

CALCULATION. For most clinical purposes, results within 1 or 2 volumes per cent of the true values for CO_2 capacity may be obtained by multiplying the observed volume (uncorrected) by 100 and subtracting 12. More accurate results are obtained by means of the table on p. 700, in which the observed volume (corrected for pressure) is directly transposed into ml. of carbon dioxide chemically bound by 100 ml. of plasma. The value obtained from this table must be multiplied by 1.017 to correct for CO_2 reabsorbed by the solution after release of pressure. The barometer reading and room temperature are taken at the time of the determination. For convenience in the calculation, values are given below for the ratio $\frac{B}{760}$ over the range usually encountered.

In case the volume of plasma taken for estimation of carbon dioxide content was 0.5 ml., the observed volume of gas is multiplied by 2 before it is used to calculate the volume per cent of carbon dioxide bound.

Interpretation. The carbon dioxide capacity of the plasma as determined by this method appears to indicate not only the alkaline reserve of the blood but also that of the entire body. The average normal value for man is 65 volumes per cent of carbon dioxide. The table on p. 701 shows the range of results obtained with normal and pathological plasma, as well as the relationship of the plasma bicarbonate to acid excretion, alkali tolerance, and alveolar carbon dioxide tension.

Barometer	$\frac{B}{760}$	*Barometer*	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	764	1.005
742	0.976	766	1.008
744	0.979	768	1.011
746	0.982	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

(b) Plasma Bicarbonate (Titration Method) Van Slyke:[9] Principle. Plasma is treated with an excess of standard acid which is titrated back with standard alkali to the original pH of the plasma as drawn.

Procedure. Blood is drawn without stasis and without exposure to air into a glass syringe or tube coated with potassium oxalate and containing mineral oil (see Fig. 183). Then without exposure to air the blood is run into a tube under oil until the tube is completely filled. A one-hole rubber stopper is inserted into the tube, expelling through the hole the oil that remains over the blood. The hole is closed with a glass plug and the tube centrifuged. The plug is then taken out and as the stopper is removed from the tube, oil is allowed to run in through the hole in the stopper to cover the surface of the plasma, so that it is never exposed to air. The plasma is then transferred under oil to another tube. Simply covering the blood with oil is not sufficient to prevent loss of CO_2 during centrifuging.

A standard for the end point is prepared in accordance with Cullen's original directions for colorimetric hydrogen-ion determination. 20 ml. of neutral 0.9 per cent NaCl containing 7 drops of 0.03 per cent phenol red is placed in a round flask of about 100 ml. capacity and covered with a layer of mineral oil. 1 ml. of the plasma is then introduced under the oil and the mixture is then stirred gently with a glass rod.

Another 1-ml. sample of the plasma is transferred to a similar round flask, 5 ml. of 0.01 N HCl, which is made up in neutral 0.9 per cent NaCl, is added, and the CO_2 is removed by whirling the mixture vigorously about the flask for at least 1 minute with a rotary motion so that the solution is whirled in a thin layer about the inner wall. 10 ml. of 0.9 per cent NaCl and 7 drops of the 0.03 per cent phenol red solution are added and 0.01 N NaOH is run in from a buret, which permits readings to 0.01 ml., until the color matches that of the standard. As the end point is approached, sufficient 0.9 per cent NaCl is added to bring the volume to 20 ml.

The 0.01 N NaOH, like the 0.01 N HCl, is made up by diluting 1 volume of 0.1 N solution to 10 volumes with neutral CO_2-free 1 per cent NaCl. The use of saline solution instead of water has the advantage of preventing the formation of a permanent cloudy precipitate of globulin.

A peculiar phenomenon occurs as the end point is approached. Each drop appears to change the color past the end point, but within a few seconds the color shifts back, and it is seen that at least another drop is needed. Consequently, the final color comparison should not be made until at least 30 seconds after the last drop of 0.01 N NaOH has been added. When in doubt, it is better to overrun the end point by a drop rather than stop short of it.

Calculations. The number of ml. of 0.01 N NaOH used in the titration is subtracted from the ml. required to neutralize to the same indicator 5 ml. of the 0.01 N HCl used. The number is approximately 5 but usually varies from it slightly because of difference in factors of acid and alkali and because of the calibration error of the 5-ml. pipet used in measuring the acid. The maximum accuracy is obtained by performing a preliminary titration on 5 ml. of the acid plus 15 ml. of distilled water, using the same pipet, indicator, and end point as in the plasma titration. The titration result represents ml. of 0.01 M $NaHCO_3$ per ml. of plasma and it is transformed into terms of molecular concentration of $NaHCO_3$ by merely dividing by 100. If the $NaHCO_3$ molecular concentration is multiplied by 2240 or the number of ml. of 0.01 N HCl used in the titration by 22.4, the volume per cent of bicarbonate CO_2 in the plasma is obtained and the results can thus be compared with those obtained by the CO_2 method.

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[9] Van Slyke: *J. Biol. Chem.*, 52, 495 (1922).

The standard 0.01 N NaOH must be protected from atmospheric CO_2 and kept in paraffined bottles to prevent solution of alkali from the glass.[10] The buret should be filled with fresh solution each day. The carbonate-free solution is made by dissolving the NaOH in an equal volume of H_2O. On standing, the Na_2CO_3 settles to the bottom. 2.75 ml. of the clear supernatant solution are diluted to 5 liters and standardized by titration with neutral red against 0.01 N HCl. It is preferable to run the acid into the alkali as the color change occurs without the time lag observed when alkali is added to acid.

Interpretation. The results agree closely with those of the CO_2 capacity method over the range of bicarbonate concentrations (0.03 to 0.01 M) ordinarily encountered in man, even in severe acidosis. Below this range the titration continues to give accurate results, while the CO_2 capacity method gives somewhat higher values. For clinical and most experimental purposes, however, it appears that the two methods give results so nearly identical that they may be used interchangeably.

3. Hydrogen-ion Concentration of Blood:

(a) COLORIMETRIC METHOD OF CULLEN[11] AS MODIFIED BY HAWKINS:[12] PRINCIPLE. Blood is collected with precautions against loss of CO_2. The plasma is diluted with saline solution containing phenol red and the color obtained compared with that of standard phosphate mixtures of known hydrogen-ion concentration.

***Procedure.* Prepare two test tubes of the same diameter as have those used to contain the standards (see below), one containing 5 ml. of 0.9 per cent sodium chloride solution and the other 5 ml. of saline indicator solution,[13] each covered with a 1-cm. layer of neutral mineral oil. Connect a Luer needle adapter, by means of a short piece of rubber tubing, to a 1-ml. Mohr pipet. Without stasis, insert the needle into the subject's vein, attach the adapter, and allow the blood to fill the pipet past the zero mark. (A tourniquet may be used to aid in finding the vein, but the blood should flow into another tube a few seconds after the release of the tourniquet, so that stasis is absent when the adapter is inserted into the needle.) Withdraw the needle from the vein and at once disconnect the rubber tubing from the pipet. Wipe the tip and introduce into each tube, under the oil, 0.4 ml. of the blood. Stir the contents carefully with a fine glass rod.**

---

[10] *Testing Standard 0.01 N NaOH for Carbonate.* The solutions should be made up using only boiled water, be kept in paraffin-lined bottles, and be protected from atmospheric CO_2 by soda-lime tubes. They should be tested for carbonate as follows:

To 5 ml. of 0.01 N HCl in a 200-ml. round flask, add from a freshly filled buret about 4.8 ml. of the 0.01 N NaOH to be tested and 0.3 ml. of neutral red solution. The mixture should be strongly acid to the indicator. The solution is rotated for 1 minute in the flask to permit the escape of CO_2, and is then transferred to a 50-ml. Erlenmeyer flask and titrated as in plasma analyses, the total amount of 0.01 N NaOH required to give the end point being noted.

A duplicate titration is performed in the same way except that there is no agitation to remove carbon dioxide, the 0.01 N HCl plus 20 ml. of water being placed directly in the 50-ml. Erlenmeyer flask, and the 0.01 N NaOH being added with a minimum of stirring. If there is no carbonate in the standard NaOH solution the two titrations give identical results. The difference should preferably not exceed 0.1 ml., and if it exceeds 0.2 ml. the alkali should not be used.

[11] Cullen: *J. Biol. Chem.*, **52**, 501 (1922).

[12] Hawkins: *J. Biol. Chem.*, **57**, 493 (1923).

[13] Prepare fresh for each determination. Add 2.1 ml. of 0.03 per cent phenol red solution to 100 ml. of 0.9 per cent sodium chloride solution. Adjust to approximately pH 7.4 by stirring with a fine glass rod dipped into 0.02 N alkali or acid, as the case may be.

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA*

Observed vol. gas $\times \frac{B}{760}$	*Ml. of* CO_2 *reduced to 0° 760 mm. bound as bicarbonate by 100 ml. of plasma*				*Observed vol. gas* $\times \frac{B}{760}$	*Ml. of* CO_2 *reduced to 0° 760 mm. bound as bicarbonate by 100 ml. of plasma*			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.8	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

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* The temperature figures at the heads of columns represent in degrees centigrade the room temperature at which the samples of plasma are saturated with alveolar carbon dioxide and analyzed. It is assumed that both operations are performed at the same temperature. The figures have been so calculated that, regardless of the room temperature at which saturation and analysis are performed, the table gives the volume (reduced to 0°, 760 mm.) of carbon dioxide that 100 ml. of plasma are capable of binding when saturated at 20° with carbon dioxide at approximately 41 mm. tension. If the figures in the table are multiplied by 0.94 they give within 1 or 2 per cent the carbon dioxide bound at 37°.

RELATIONSHIP OF THE PLASMA BICARBONATE TO ACID EXCRETION, ALKALI TOLERANCE, AND ALVEOLAR CARBON DIOXIDE TENSION*

Condition of Subject	*Actual Bicarbonate Reserve. Plasma Bicarbonate CO_2 Reduced to 0°, 760 mm.*	*Corresponding Results of Indirect Tests for Acidosis*					
		24-hr. Excretion† of 0.1 N acid + NH_3		*Carbon Dioxide of Alveolar Air*		*Sodium Bicarbonate Required to Turn Urine Alkaline*	
		(*a*) Ml. per kg. (*b*) Approximate ml. per 60 kg. person	Reliability in diabetes	(*a*) Mm. tension (*b*) Approximate per cent	Reliability in diabetes	(*a*) G. per kg.‖ (*b*) Approximate g. for a 60-kg. person	Reliability in diabetes
	vol. per cent	*ml.*					
Normal resting adult Extreme limits of bicarbonate reserve	80–53	(*a*) 0–27 (*b*) 0–1600	Good	(*a*) 53–35 mm. (*b*) 6.8–4.7 per cent	May indicate some acidosis in its absence	(*a*) 0–0.5 (*b*) 0–30	May indicate acidosis in its absence
Mild acidosis, no pronounced symptoms	53–40	(*a*) 27–65 (*b*) 1600–4000	Good§	(*a*) 35–27 mm. (*b*) 4.7–3.6 per cent	May indicate more acidosis than is present	(*a*) 0.5–0.8 (*b*) 30–50	May indicate much more acidosis than is present
Moderate to severe acidosis. Symptoms may be apparent	40–30	(*a*) 65–100 (*b*) 4000–6000	Liable to considerable error in either direction§	(*a*) 27–20 mm. (*b*) 3.6–2.7 per cent	Good	(*a*) 0.8–1.1 (*b*) 50–65	
Severe acidosis. Symptoms of acid intoxication	Below 30	(*a*) Over 100 (*b*) Over 6000		(*a*) Below 20 mm. (*b*) 2.7 per cent	Good	(*a*) Over 1.1 (*b*) Over 65	

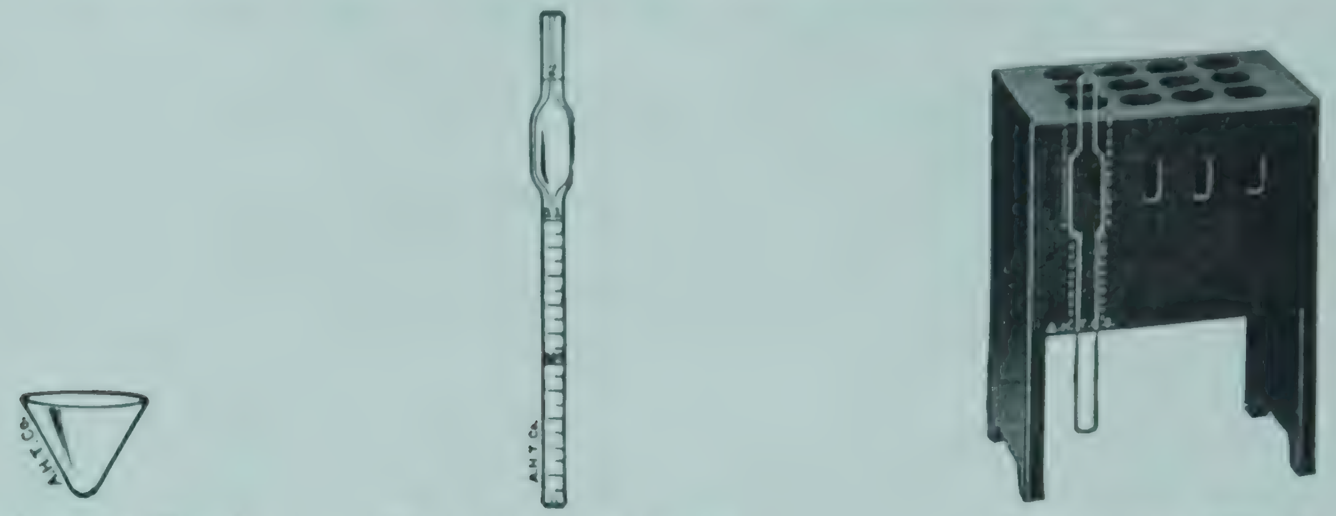
* Van Slyke: *J. Biol. Chem.*, **33**, 271 (1918).

† Measured either in 24-hour urine or in specimen from shorter period calculated to 24-hour basis.

§ After bicarbonate administration, likely to indicate more acidosis than is present.

‖ The figures tabulated in this column also indicate the doses of bicarbonate necessary to restore the alkali reserve to normal from acidosis of the severity indicated by the corresponding plasma CO_2 figures in the first column.

tubes of the same internal diameter as the bulb of the pipets. The contents of the pipet may then be transferred to the Van Slyke-Neill apparatus.



Receiver for Blood *Shock-Hastings Pipet* *Comparator Block*

FIG. 185. APPARATUS FOR SHOCK-HASTINGS METHOD.

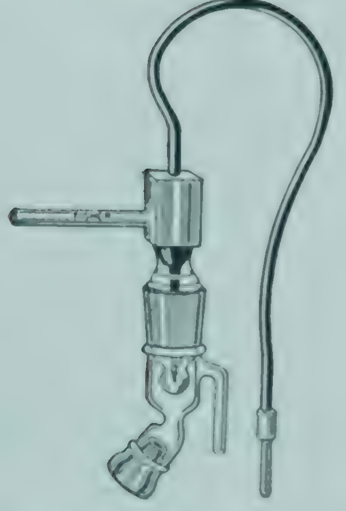


FIG. 186. GLASS-ELECTRODE ASSEMBLY FOR USE WITH BECKMAN pH METER.

Courtesy, Fisher Scientific Company, New York.

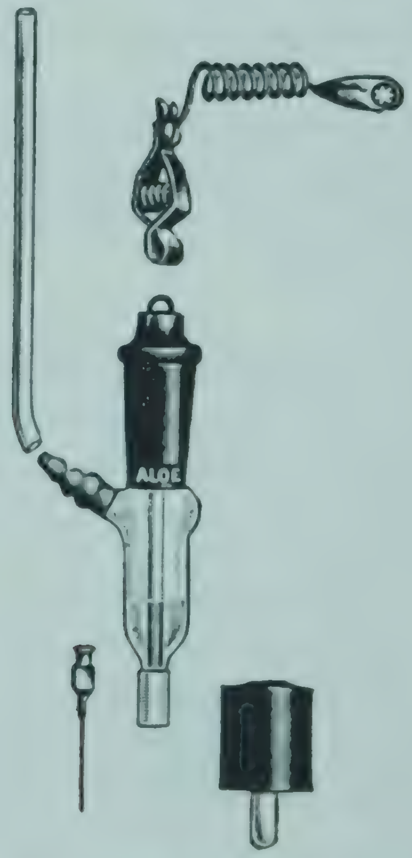


FIG. 187. GLASS ELECTRODE FOR USE WITH THE COLEMAN ELECTROMETER.

Courtesy, A. S. Aloe Company, St. Louis, Mo.

(c) ELECTROMETRIC METHOD.[16] The potentiometric method for the determination of pH has been applied to blood. The usual precautions must be observed in obtaining the sample to prevent the loss of carbon dioxide, and similar care must likewise be taken during the actual measurement. To this end, glass-electrode vessels have been devised so that the sample of blood is maintained out of contact with air. Fig. 186 shows such a glass-electrode assembly for use with the Beckman pH meter, so designed that the sample of blood can be transferred with minimum gas exchange directly from the hypodermic syringe to the electrode chamber. Another model of glass electrode, for use with the Coleman Electrometer, is shown in Fig. 187. In this assembly, the hypodermic needle is attached to the electrode chamber so that the sample of blood enters the chamber, which is then immediately made part of the electrometric circuit. With the due care ordinarily exercised in the electrometric measurement of pH, accurate values can be obtained on as little as 0.5 ml. of blood.

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[16] Craig, Lange, Oberman, and Carson: *Arch. Biochem. and Biophys.*, 38, 357 (1952).

4. Acetone Bodies. For methods of determining acetone, acetoacetic, and β-hydroxybutyric acids in the blood see the References at the end of Chapter 23.

5. Determination of Oxygen and Oxygen Capacity (or Hemoglobin) of Blood: Volumetric Methods. It is possible to determine the oxygen of the blood using the same apparatus as that employed for the CO_2 estimation (see p. 693), suitable precautions being taken in collecting the blood for analysis. The *oxygen content* of blood represents the total volume of oxygen, both physically dissolved and combined with hemoglobin, present in 100 ml. of blood in the condition in which it flows through the veins or arteries. For the determination of oxygen content it is, therefore, necessary to collect the blood so as to avoid the oxygenating effect of air, i.e., under oil. An aspirating tube of the type illustrated in Fig. 183 is suitable for this purpose.

The *oxygen combined with hemoglobin* in arterial or venous blood differs from the oxygen content in that a correction is made for free, physically dissolved oxygen. The same precautions in the collection of the sample must, of course, be taken.

The *oxygen capacity* of blood represents the volume of oxygen required to combine with all of its hemoglobin (oxyhemoglobin and reduced hemoglobin). It therefore constitutes a measure of hemoglobin since it is established that 1 g. of hemoglobin combines with 1.36 ml. of oxygen, or, in other words, each ml. of oxygen capacity represents 0.736 g. of hemoglobin. Blood for this determination may be collected without precautions to avoid contact with air, inasmuch as aeration (oxygenation) is the first step in the procedure.

The *oxygen unsaturation* of venous or arterial blood is a measure of the absolute concentration of reduced hemoglobin, and is obtained by subtracting from the oxygen capacity (total hemoglobin), the oxygen combined with hemoglobin as defined above. The distinction between this and the relative unsaturation, which is the *per cent* of total hemoglobin in the reduced state, should be kept in mind. The use of the absolute rather than the relative expression is due to the fact that cyanosis is regarded as the result of an increased absolute concentration of the bluish reduced hemoglobin.

Determination of Oxygen Capacity. **Stir the special oxygen reagent (see p. 716) to obtain emulsification of the caprylic alcohol. Introduce 7.5 ml. into cup *b* (Fig. 182), and deaerate by shaking in the evacuated extraction chamber. Mix the blood thoroughly with a stirring rod, and transfer to a 250-ml. separatory funnel in which it is completely aerated by rotating in a thin layer over the interior surface of the funnel. (For oxygen determinations other than capacity, simply mix the blood under the oil and omit aeration.) Force 6 ml. of the gas-free reagent into the cup of the apparatus. By means of a differential pipet (graduated to deliver between marks, Fig. 190), transfer exactly 2 ml. of the blood directly into the extraction chamber by**

keeping the tip of the pipet immersed to the bottom of the reagent in the cup and regulating the admission by the finger or stopcock on the pipet and cock *e* of the apparatus. Never allow more than a few ml. of blood to accumulate in the cup. An Ostwald transfer pipet may be used for this purpose, the final drop of blood being forced out of the immersed tip by expanding the air in the pipet with the heat of the hand, keeping the mouth of the pipet closed. Admit the remaining blood and all but 1 ml. of the reagent from cup *b* into the extraction chamber. With a medicine dropper discard the excess reagent and introduce a few drops of mercury as a seal. Evacuate and shake until the volume of extracted gas ($O_2 + CO_2 + N_2$) is constant. This will be found to consume from 5 to 10 minutes and should be determined by measuring the gas at atmospheric pressure. Create a slight negative pressure within the pipet by bringing the level of solution to the 2-ml. graduation. Rinse cup *b* with distilled water and introduce 0.5 ml. of a 2 per cent NaOH solution previously deaerated. By means of the negative pressure within the pipet, admit this solution slowly, followed by a thin stream of mercury, which serves to break the column of NaOH which usually forms in the capillary of the pipet. Allow 1 minute for drainage and bring the gas ($O_2 + N_2$) to atmospheric pressure as described in the determination of CO_2. Read the volume of gas, the temperature, and the barometric pressure.

CALCULATIONS.

V = observed volume of gas ($O_2 + N_2$)
t = temperature in ° C.
B = barometric pressure in mm. Hg
w = tension of aqueous vapor (see p. 709)

$$\text{Volume per cent } O_2 \text{ capacity} = \left(\frac{(B - w)}{760(1 + 0.00367t)} \times \frac{100V}{2}\right) - 2.1$$

Values for the temperature and pressure correction factor are given in the table on p. 707. The following equation may also be used:

$$\text{Volume per cent } O_2 \text{ capacity} = \left(\frac{17.9(B - w)V}{t + 273}\right) - 2.1$$

The value 2.1 in these equations corrects for oxygen and nitrogen physically dissolved at atmospheric pressure. For oxygen content subtract 1.36 (per cent N_2) instead of 2.1. For O_2 combined with hemoglobin subtract 1.7 or 1.5 for arterial or venous blood respectively, which corrects for free O_2 and N_2 at arterial or venous tension. O_2 unsaturation is obtained by subtracting O_2 combined with hemoglobin from O_2 capacity.

Hemoglobin (g. per 100 ml.) = 0.736 × volume per cent O_2 capacity.

For colorimetric determination of hemoglobin, see p. 610.

6. Determination of Carbon Monoxide in Blood—Volumetric Method of Van Slyke and Associates:[17] ***Principle.*** A more exact quantitative procedure for the estimation of carbon monoxide than the colorimetric method given in Chapter 22 is a volumetric method based on the experiments of Van Slyke and his associates. The mixed gases are extracted from the sample of blood in a Van Slyke pipet (Fig. 182), the oxygen and carbon dioxide are absorbed, and the carbon monoxide estimated in the residual gas either by absorption with Winkler's reagent or by correction for the nitrogen.

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[17] Van Slyke and Salvesen: *J. Biol. Chem.*, **40**, 103 (1919); Van Slyke and Stadie: *J. Biol. Chem.*, **49**, 1 (1921); Van Slyke and Neill: *J. Biol. Chem.*, **61**, 523 (1924). Adapted by Bernard L. Oser.

TABLE OF FACTORS FOR CALCULATION

Temperature	$f = \frac{B - w}{760(1 + 0.00367t)}$ factor by which gas measured moist at t°, B mm., is reduced to 0°, 760 mm.*	α'_{CO_2}	Air† measured at room temperature and pressure, dissolved by		$1.017f\left(1 + \frac{S}{50 - S}\alpha'_{CO_2}\right)$ factor by which the volume of CO_2 obtained after 1 extraction is multiplied in order to obtain the volume of CO_2 reduced to 0°, 760 mm., contained in the solution analyzed	
			2.5 ml. H_2O	5 ml. H_2O	S = 2.5 ml.	S = 5.0 ml.
° *C.*			*ml.*	*ml.*		
15	$0.932 \times \frac{B}{760}$	1.075	0.052	0.105	$1.002 \times \frac{B}{760}$	$1.061 \times \frac{B}{760}$
16	$0.928 \times \frac{B}{760}$	1.043	0.051	0.101	$0.995 \times \frac{B}{760}$	$1.053 \times \frac{B}{760}$
17	$0.924 \times \frac{B}{760}$	1.015	0.050	0.100	$0.989 \times \frac{B}{760}$	$1.046 \times \frac{B}{760}$
18	$0.919 \times \frac{B}{760}$	0.989	0.049	0.098	$0.983 \times \frac{B}{760}$	$1.038 \times \frac{B}{760}$
19	$0.915 \times \frac{B}{760}$	0.966	0.048	0.096	$0.978 \times \frac{B}{760}$	$1.030 \times \frac{B}{760}$
20	$0.910 \times \frac{B}{760}$	0.942	0.047	0.095	$0.972 \times \frac{B}{760}$	$1.022 \times \frac{B}{760}$
21	$0.906 \times \frac{B}{760}$	0.919	0.046	0.093	$0.966 \times \frac{B}{760}$	$1.015 \times \frac{B}{760}$
22	$0.901 \times \frac{B}{760}$	0.896	0.045	0.091	$0.960 \times \frac{B}{760}$	$1.008 \times \frac{B}{760}$
23	$0.897 \times \frac{B}{760}$	0.873	0.045	0.090	$0.954 \times \frac{B}{760}$	$1.001 \times \frac{B}{760}$
24	$0.892 \times \frac{B}{760}$	0.850	0.044	0.088	$0.948 \times \frac{B}{760}$	$0.993 \times \frac{B}{760}$
25	$0.888 \times \frac{B}{760}$	0.828	0.043	0.086	$0.942 \times \frac{B}{760}$	$0.986 \times \frac{B}{760}$
26	$0.883 \times \frac{B}{760}$	0.808	0.042	0.084	$0.936 \times \frac{B}{760}$	$0.978 \times \frac{B}{760}$
27	$0.878 \times \frac{B}{760}$	0.789	0.041	0.083	$0.931 \times \frac{B}{760}$	$0.971 \times \frac{B}{760}$
28	$0.873 \times \frac{B}{760}$	0.772	0.040	0.081	$0.924 \times \frac{B}{760}$	$0.964 \times \frac{B}{760}$
29	$0.868 \times \frac{B}{760}$	0.755	0.040	0.080	$0.918 \times \frac{B}{760}$	$0.957 \times \frac{B}{760}$
30	$0.863 \times \frac{B}{760}$	0.738	0.039	0.078	$0.912 \times \frac{B}{760}$	$0.950 \times \frac{B}{760}$

---

* To calculate O_2 or hemoglobin when $O_2 + N_2$ volume is measured, multiply gas volume by f, to reduce to 0°, 760 mm., and by such factor as is necessary (100 when 1 ml. of blood is used, 50 when 2 ml. are used) to bring results to volume per cent basis. Then for

a, O_2 content, subtract	1.36 vol. per cent N_2
b, O_2 bound by hemoglobin in venous blood, subtract	1.5 vol. per cent N_2 + dissolved O_2
c, O_2 bound by hemoglobin in arterial blood, subtract	1.7 vol. per cent N_2 + dissolved O_2
d, O_2 bound by hemoglobin in blood saturated with air at 20° C., subtract	2.1 vol. per cent N_2 + dissolved O_2

Per cent of normal hemoglobin (Haldane scale) $= \frac{100d}{18.5} = 5.41d$

Grams of hemoglobin per 100 ml. of blood $= 0.736d$

Per cent of total hemoglobin saturated with $O_2 = \frac{100b}{d}$ or $\frac{100c}{d}$

Volumes per cent O_2 unsaturation $= d - c$ or $d - b$

† The dissolved air is given as measured at room temperature. It is subtracted from the air + CO_2 volume, measured after one extraction of plasma or aqueous carbonate solution, in order to obtain the CO_2, which is then multiplied by $1.017f\left(1 + \frac{S}{50 - S}\alpha'_{CO_2}\right)$ in order to obtain the total volumes per cent of CO_2 in the solution analyzed. When whole blood is analyzed, the air correction cannot be used because of the O_2 present, and the CO_2 must be determined by absorption with NaOH solution. The volume of gas absorbed is then multiplied by the above factor.

The factor 1.017, being empirical, may vary slightly for different apparatus.

Procedure. Blood should be collected under oil in an oxalated tube. A special CO reagent[18] is required which differs from the O_2 reagent in containing lactic acid as well as more potassium ferricyanide. The reagent (7.5 ml.) is made gas-free and the mixed gases are extracted from 2 ml. of blood exactly as described under the determination of oxygen capacity (p. 705). Longer shaking may be required to arrive at constant volume, since CO is not so readily dissociated from its combination with hemoglobin. After the extraction, a negative pressure is created in the extraction chamber by raising the solution level to a point 2 to 3 cm. below the 2.5-ml. graduation. About 0.5 ml. of mineral oil is introduced into the cup and below this 1 ml. of alkaline pyrogallate reagent.[19] The reagent is admitted until its level reaches the capillary of the cup, and the column that forms in the graduated capillary is broken by means of a stream of mercury as described in the determination of oxygen capacity. A few minutes are allowed for drainage, the gas is restored to atmospheric pressure, and the volume read. The absorption of CO_2 and O_2 is repeated until the residual volume is constant. The CO may be estimated from this volume reading, the temperature, and barometric pressure.

If it is desired to determine the CO directly by absorption, proceed from this point by first drawing the mixed solutions in the pipet into reservoir *d*. This is to avoid mixing the alkaline pyrogallate solution with Winkler's reagent[20] to be used. Establish a slight negative pressure within the extraction chamber as described above. Rinse the delivery cup with distilled water and introduce into the pipet 0.5 ml. of Winkler's reagent. Absorption of CO takes place rapidly and the final gas volume should be read at once.

A method of magnifying small volumes of gas by reducing the pressure a definite amount below atmospheric is described by Van Slyke and Stadie.[21]

Larger samples of blood may be used, the amounts of reagents being proportionately increased.

CALCULATIONS.

V_1 = Observed volume of gas from 2 ml. of blood, after absorption by alkaline pyrogallate solution

V_2 = Observed volume after absorption by Winkler's reagent

B = Barometric pressure in mm. of Hg

w = Tension of aqueous vapor in mm. of Hg (see table on p. 709)

t = Temperature in ° C.

$$\text{Volume per cent CO} = \left(\frac{(B - w)}{760(1 + .00367t)} \times \frac{100V_1}{2}\right) - 1.36$$

or

$$\text{Volume per cent CO} = \left(\frac{(B - w)}{760(1 + .00367t)}\right) \frac{100(V_1 - V_2)}{2}$$

---

[18] *Special CO Reagent.*

Saponin	3.0 g.
Potassium ferricyanide	8.0 g.
Lactic acid c.p	4.0 ml.
Caprylic alcohol	3.0 ml.
Distilled water to	1000 ml.

[19] *Alkaline Pyrogallate Reagent.* Prepare a solution of potassium hydroxide by dissolving 160 g. in 130 ml. of water. In 200 ml. of this solution dissolve 10.0 g. of pyrogallic acid.

[20] *Winkler's Reagent.* Cuprous chloride 40 g., ammonium chloride 50 g., distilled water to 150 ml.

For use mix this solution with ammonium hydroxide (sp. gr. 0.9) in the proportion of 3:1.

[21] Van Slyke and Stadie: *J. Biol. Chem.*, 49, 1 (1921).

Values for the temperature and pressure correction factor may be found in the table on p. 707.

VAPOR TENSION OF WATER

° *C.*	*mm. Hg*	° *C.*	*mm. Hg*
10	9.1	20	17.4
11	9.8	21	18.5
12	10.4	22	19.6
13	11.1	23	20.9
14	11.9	24	22.2
15	12.7	25	23.5
16	13.5	26	25.0
17	14.4	27	26.5
18	15.3	28	28.1
19	16.3	29	29.7
		30	31.5

MANOMETRIC METHODS OF ANALYSIS OF GASES IN BLOOD AND OTHER SOLUTIONS[22]

Principle. The manometric methods of gas analysis differ from the more commonly used volumetric methods in that the latter involve reading the volume with the gas under a definite (usually atmospheric) pressure, whereas by manometric methods the pressure required to keep the gas at a fixed volume is observed. The advantages of this procedure are that a much lower degree of error is attainable, since in the former method the error in volume reading is many times greater than the error in reading of barometric pressure; calculation is greatly simplified, as barometric pressure and corrections for vapor tension and capillary attraction of mercury do not enter in; smaller quantities of material may be used; and accuracy is attainable over a wide range of gas concentration.

Apparatus. The apparatus consists of a short pipet with the upper stem closed by a stopcock, the lower connected with a glass tube. The latter descends, then turns to connect with a leveling bulb and a closed mercury manometer. The pipet is calibrated at two points to hold a ml. of gas for pressure measurement and A ml. of total volume, respectively, as shown in Fig. 188.

For analysis, the sample of blood or other solution is introduced into the chamber over mercury, together with the reagents to free the desired gases from combination. A Torricellian vacuum is obtained, as in the "volumetric" apparatus, by lowering the leveling bulb, and the gases are extracted from solution by 2 or 3 minutes' shaking. The gas volume is then reduced to a ml. by admission of mercury and the reading p_1 is made on the manometer. The gases are either ejected or are absorbed by proper reagents, and the reading p_2 is taken, with the same gas volume. The partial pressure P of the gas at a ml. volume is then $P = p_1 - p_2$ mm. of mercury, from which the gas volume at 0°, 760 mm. may be calculated. Methylene or ethylene glycol or glycerol may be used as a dehydrating agent to moisten the upper part of the manometer tube.

---

[22] Van Slyke and Neill: *J. Biol. Chem.*, **61**, 523 (1924); Van Slyke: *J. Biol. Chem.*, **73**, 121 (1927). In addition to the methods here to be described, these papers give the details of the technique for the combined determination of all the gases in a sample of blood: methods for micro-analyses of fractions of a ml. of blood; for determination of gases in liquids saturated at high tensions; for determination of dissolved gases in water; and for the use of the apparatus in air and general gas analyses. Principles and numerous applications of the manometric methods are completely described in Peters and Van Slyke: *Quantitative Clinical Chemistry*, Vol. 2, Baltimore, The Williams & Wilkins Co., 1932.

The extraction chamber differs from that of the "volumetric" apparatus in being calibrated at only three points, viz., 0.5, 2.0, and 50 ml. The mercury seal around the rubber joint at the bottom of the extraction chamber illustrated in Fig. 189 can be replaced by special heavy-walled rubber tubing. A mechanical shaker is provided. Air which diffuses through the rubber tubing of the leveling bulb is expelled through

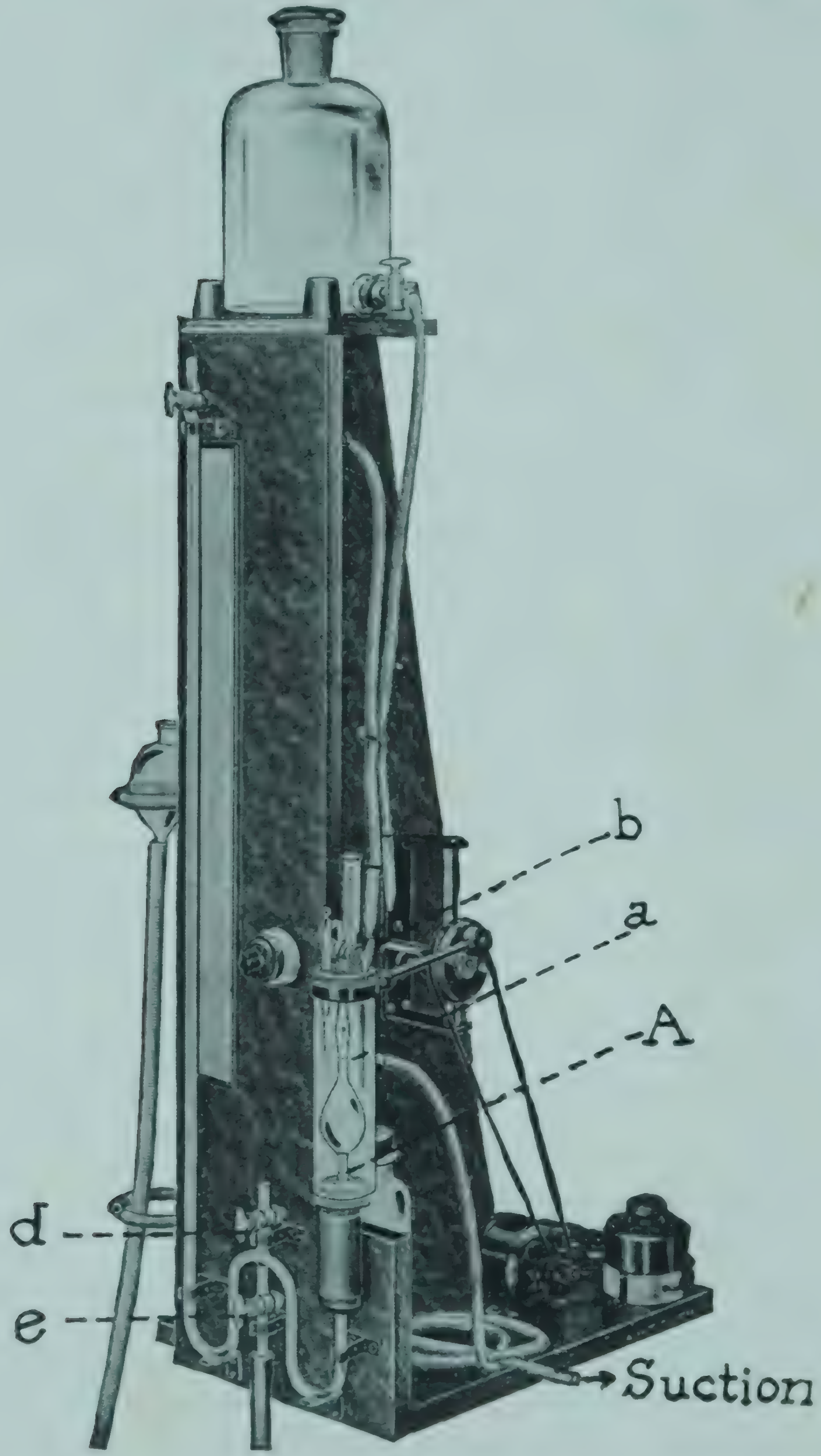

FIG. 188. VAN SLYKE AND NEILL PORTABLE MANOMETRIC GAS-ANALYSIS APPARATUS.

stopcock *d*. The stopcock at the bottom of the manometer permits withdrawal of the mercury.

The bottle on top of the frame holds distilled water. The lower bottle is to receive waste solutions drained out of the chamber after analyses. The most rapid and convenient way to transfer solution to the waste bottle is to force it up into the cup above the chamber and then draw it over into the bottle by suction. If suction is not available, a narrow rubber drain can be run from the curved outlet capillary to the bottle.

For calibration of the apparatus and a method of constructing a simple but not necessarily inaccurate modification of the manometric apparatus, see the original papers.

Measuring Samples. In order to attain the advantage of precision offered by the manometric apparatus, accuracy in collecting and measuring samples is essential. Blood should be either (1) drawn directly from the vein, without stasis, into the differential pipet from which it is to be delivered into the extraction chamber, or, better (2), collected under oil in an oxalated tube from which it can be drawn as needed. The corpuscles should be uniformly distributed with a fine stirring rod before each sample is withdrawn. A heavy-walled Ostwald pipet graduated to deliver between two marks ("differential" pipet) and preferably provided with a stopcock should be used (see Fig. 190).

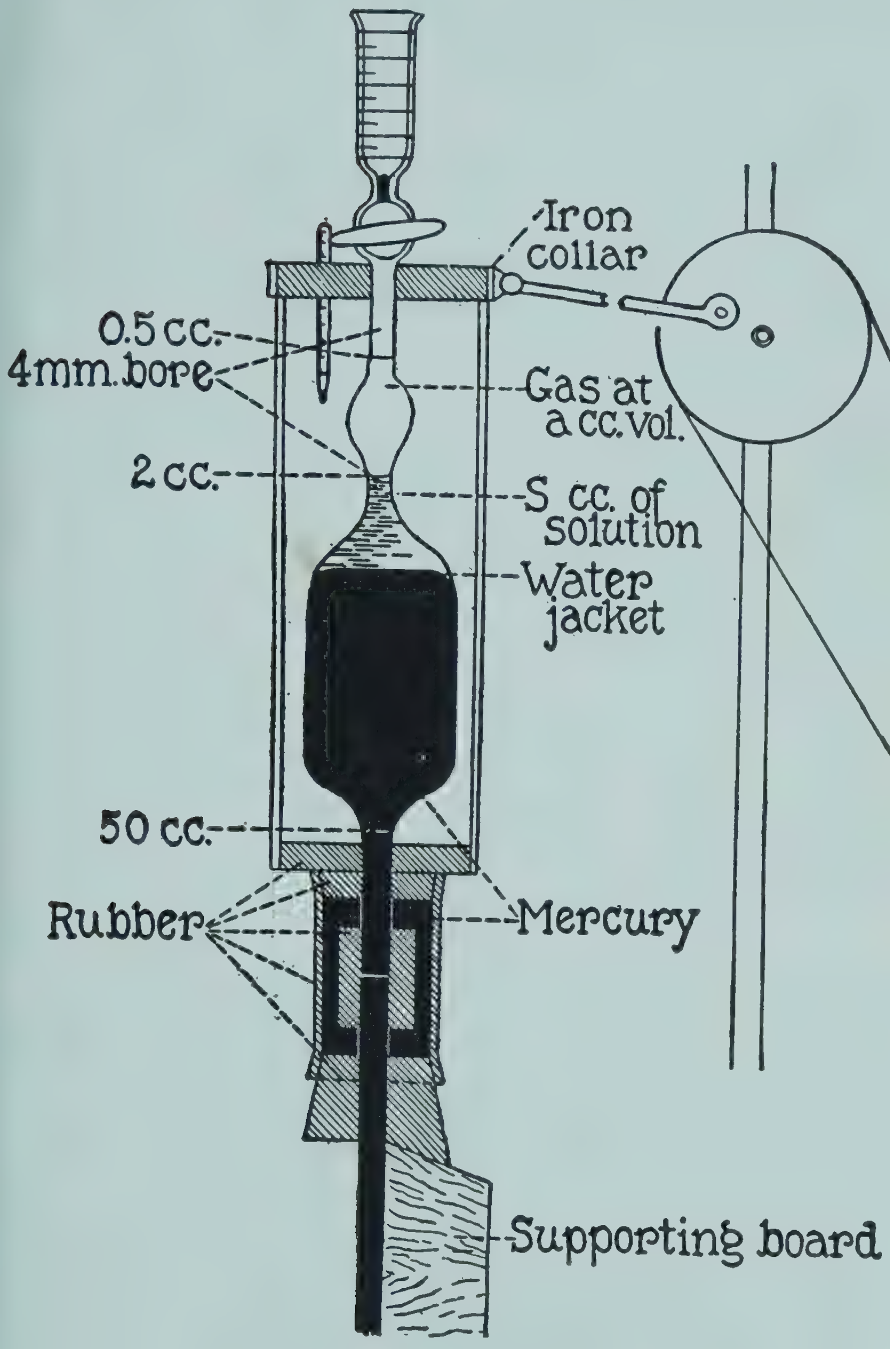

FIG. 189. EXTRACTION CHAMBER.

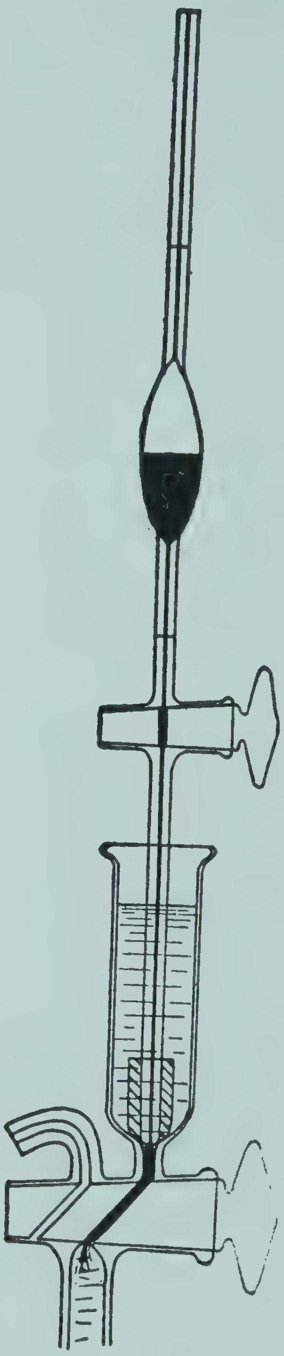

FIG. 190. OPERATION OF DELIVERY PIPET.

Blood must be admitted at a rate slow enough to permit clear drainage of the pipet. Smooth delivery is usually obtained by using cock *e* or the cock of the pipet. Amounts of solution of 2 ml. or more may be run in under 1 ml. of mercury in the cup, the mercury then washing out the capillary. For smaller amounts, wash solution is necessary for complete transfer. An ordinary transfer pipet may be used, the final drop of blood being expelled by keeping the tip immersed, closing the mouth of the pipet with the index finger, and grasping the bulb with the warm palm of the other hand. The expanding air drives out the remaining blood.

Cleaning the Apparatus. Preceding each analysis, the apparatus is cleaned by introducing 10 or 15 ml. of approximately 0.01 *N* lactic acid, shaking for 15 or 20 seconds in the evacuated chamber, and ejecting the extracted gas and solution. Adherent solution introduces no error in the subsequent analysis.

Factors for Calculation of Volumes Per Cent of O_2, CO and N_2 from Pressures in 50-ml. Apparatus*

Temperature	*Sample* = 0.2 ml. *S* = 2.0 ml. *a* = 0.5 ml. *i* = 1.00	*Sample* = 1 ml. *S* = 3.5 ml.		*Sample* = 2 ml. *S* = 7 ml.	
		a = 0.5 ml. *i* = 1.00	*a* = 2.0 ml. *i* = 1.00	*a* = 0.5 ml. *i* = 1.00	*a* = 2.0 ml. *i* = 1.00
° C.					
15	0.312	0.0623	0.2493	0.0317	0.1251
16	10	21	85	15	46
17	09	19	78	14	42
18	08	17	68	12	37
19	07	15	59	11	32
20	07	13	50	09	28
21	06	10	41	08	24
22	05	08	32	06	19
23	03	06	23	05	15
24	02	04	14	03	10
25	01	02	06	02	06
26	00	00	0.2398	01	02
27	0.299	0.0598	90	0.0299	0.1198
28	98	96	82	98	93
29	97	93	74	96	89
30	96	92	66	95	85
31	95	90	58	94	81
32	94	88	50	92	77
33	93	86	42	91	73
34	92	83	33	90	69

* If calibration of an apparatus shows a value of *a* significantly different from the 0.500 or 2.000 ml. in the column heading, the factors in the column are corrected by multiplying them by $\frac{a}{0.500}$ or $\frac{a}{2.000}$.

Occasionally the apparatus should be kept filled overnight with chromic-sulfuric acid mixture, admitted through cock *b* by regulation of cock *e*. The latter is kept closed but the former open to allow the escape of gas.

Testing for Leaks. Test for leaks by introducing *S* ml. of water (Fig. 189) and extracting dissolved air for two minutes. Reduce the gas volume to *a* and read the pressure. If the temperature is constant, the pressure should not increase when this extraction is repeated.

FACTORS BY WHICH MILLIMETERS P_{CO_2} ARE MULTIPLIED TO GIVE VOLUMES PER CENT CO_2 IN SOLUTION ANALYZED—50-ML. APPARATUS*

Temperature	*Sample* = 0.2 ml.	*Sample* = 1.0 ml.					
	S = 2.0 ml. *a* = 0.5 ml. *i* = 1.037	*S* = 2.0 ml.		*S* = 3.5 ml.		*S* = 7.0 ml.	
		a = 0.5 ml. *i* = 1.037	*a* = 2.0 ml. *i* = 1.017	*a* = 0.5 ml. *i* = 1.037	*a* = 2.0 ml. *i* = 1.017	*a* = 0.5 ml. *i* = 1.037	*a* = 2.0 ml. *i* = 1.017
° C.							
10	0.3454	0.0691	0.2710	0.0718	0.2818	0.0789	0.3097
11	37	87	0.2696	14	00	83	70
12	19	84	83	09	0.2783	76	44
13	03	81	70	05	67	70	20
14	0.3386	77	56	01	50	64	0.2996
15	70	74	44	0.0697	35	58	74
16	54	71	31	93	19	52	50
17	38	68	18	89	04	46	28
18	22	64	06	86	0.2690	41	06
19	07	61	0.2594	82	75	36	0.2886
20	0.3292	58	83	78	62	31	66
21	78	56	72	75	48	26	48
22	63	53	60	71	34	21	28
23	48	50	48	68	20	16	08
24	34	47	37	65	07	11	0.2790
25	20	44	26	61	0.2594	07	72
26	06	41	15	58	81	02	53
27	0.3193	39	05	55	69	0.0698	36
28	79	36	0.2494	52	57	93	20
29	66	33	84	49	45	89	04
30	53	31	74	46	33	85	0.2688
31	40	28	64	43	22	82	74
32	28	26	54	40	11	78	59
33	15	23	44	37	00	74	44
34	03	21	35	34	0.2489	71	30

* Van Slyke and Sendroy: *J. Biol. Chem.*, **73**, 127 (1927).

To obtain factor for a sample other than 1 ml., divide the above factors for 1 ml. by the ml. of sample analyzed: e.g., for a 2-ml. sample the factors are one-half of those for 1 ml.

To calculate ml. of CO_2, measured at 0°, 760 mm., in the actual portion of solution analyzed, use the above volume per cent factors for 1-ml. samples divided by 100.

Lubrication. Cocks *b* and *e* must turn smoothly but not leak. A thin layer of petrolatum is first applied, followed by a rubber paste (made by dissolving 1 part of unvulcanized rubber in 5 parts of petrolatum, with heat), using relatively less of the first coating in warm weather.

Determination of Correction, *c*, for Manometer Depression Caused by Introduction of Absorbent Solution. The introduction of absorbent solution causes a lowering of the mercury meniscus in the chamber and hence in the manometer, by increasing the volume of fluid between the mercury and the *a* mark at the moment of reading (Fig. 188). This necessitates for the p_2 reading a correction, which is determined by blank analyses. The shape of the apparatus causes the area of the meniscus of the mercury in the chamber to vary according to the values of S and a. The value of c for 1 ml. of added solution may accordingly be from 1 to 4 mm., depending on the shape of the chamber and the volume of S.

Dilute absorbents (N NaOH) or hydrosulfite (20 per cent solution) have no significant effect on the vapor pressure in the chamber. Special precautions are required for alkaline pyrogallate solution (p. 708) because of the strong KOH.

When the final manometer reading is obtained after expulsion of the gases instead of after addition of an absorbing solution, *c*, of course, is zero.

CALCULATION. The general equation for calculating total gas content of a solution from the volume of gas extracted in an evacuated chamber of definite volume, as developed by Van Slyke and Stadie,[23] has been subjected to certain modifications and made adaptable to manometric calculations.

Vapor pressure is eliminated as a factor since it is the same for both pressure readings and cancels out in the equation $P = p_1 - p_2$. An *i* correction for reabsorption of extracted gas during the release of the vacuum is required for CO_2 just as in volumetric measurements, and amounts to about 1.014. For less soluble gases (O_2, N_2, CO) it is practically 1.000, reabsorption being negligible. A correction is also included for the effect of temperature on the specific gravity of mercury.

The use of the rather involved final equation is expedited by the tables constructed by the authors, which give directly the factors by which P, read at any given temperature under the conditions ordinarily employed, must be multiplied to obtain the volumes per cent of gas (see the tables on pp. 712 and 713). To express results as millimoles per liter, either use the table of millimole per liter factors given in the original papers, or divide volumes per cent of gas by 2.24 or in the case of CO_2 by 2.226.

$$\text{Volumes per cent gas} = P \times \text{vol. per cent factor}$$

$$\text{mM. gas per liter} = \frac{P \times \text{vol. per cent factor}}{2.24}$$

Determination of CO_2 in Blood or Plasma.[24] **The apparatus having been cleaned (see p. 712), a drop of caprylic alcohol is drawn into the capillary above cock *b*, and 2.3 ml. of CO_2-free water per ml. of blood or plasma to be added are put into the cup. Stopcock *b* is closed, with *e* open. The blood or plasma is delivered beneath the layer of water in the cup from a pipet as described under "Measuring Samples." After the delivery of the sample, the residue of blood in the cup is run into the chamber below, followed by the water layer. Finally, 0.2 ml. of CO_2-free 0.1 N lactic acid per ml. of blood or plasma is added. Stopcock *b* is then sealed with a drop of mercury. The CO_2 is liberated by lowering the leveling bulb until the surface of the mercury has fallen to the *A* mark, closing cock *e*, and shaking the mixture for 3 minutes. The extracted gas is reduced to 2 ml. (*a*), the admission of mercury being regulated by stopcock *e* and the leveling bulb. If the fluid meniscus passes this point, readjustment must be made by first bringing the mercury meniscus to the *A* mark and equilibrating for a minute. Otherwise more reabsorption of CO_2 will take place than is provided for in the calculation. The adjustment being correct, the manometer is tapped with the finger, and the height of the mercury column read (p_1 mm.).**

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[23] *J. Biol. Chem.*, **49**, 30, 31 (1921).

[24] Shohl determines both CO_2 and pH on 0.1 to 0.2 ml. of plasma. See *J. Biol. Chem.*, **83**, 759 (1929).

The variable amounts of O_2 extracted from whole blood make it necessary to determine CO_2 by absorption. This custom is also followed in plasma analyses, though it may be avoided by correcting for extracted air. The absorbent solution, 1 N NaOH[25] is admitted under reduced pressure. After measuring p_1 the pressure is diminished so that a space of several ml. is left between the water meniscus and the lower *a* mark. Next, 2 ml. of gas-free alkali is measured into the cup from the reservoir, avoiding reabsorption of air, and 1 ml. of alkali is admitted into the chamber. Absorption is complete in 30 seconds.

In analyses of serum or aqueous solutions of carbonates, the CO_2 may be absorbed with 0.2 ml. of 5 N NaOH. O_2 and N_2 are sufficiently insoluble in this medium to make deaeration unnecessary. Only slight negative pressure is required to admit it. Air-free 1 N NaOH must be used for whole blood.

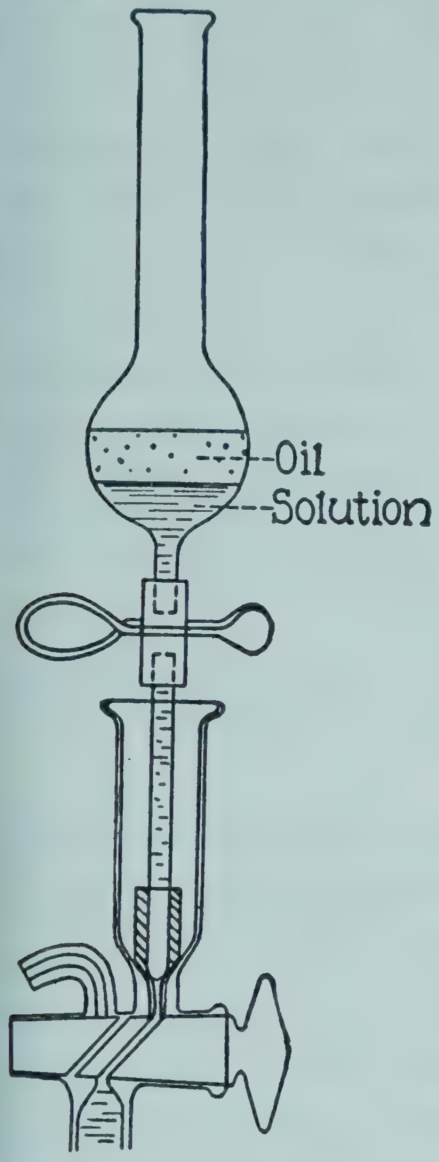

FIG. 191. USE OF CALCIUM CHLORIDE TUBE RESERVOIR.

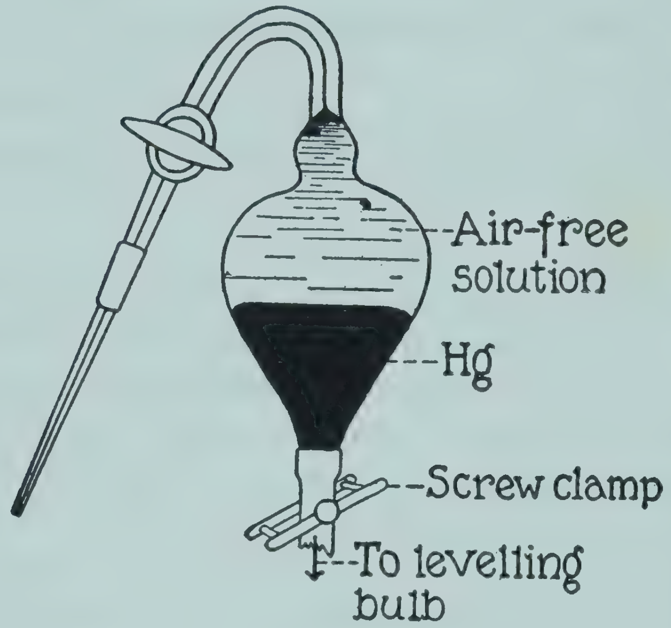

FIG. 192. BULB FOR PREPARING AND STORING GAS-FREE SOLUTIONS.

After absorption of CO_2 cock *b* is sealed with mercury and the meniscus of the solution in the chamber is lowered a little below the *a* mark. Mercury is readmitted from cock *e* until the solution meniscus is again on the *a* mark. Cock *e* is closed and the reading of the manometer is taken (p_2 mm.). The CO_2 pressure P_{CO_2} is

$$P_{CO_2} = p_1 - p_2 - c$$

where *c* is the correction discussed on p. 713. This correction is determined in a blank analysis in which *S* ml. of water, made alkaline with 2 or 3 drops of normal NaOH, are extracted in the apparatus, p_1 and p_2 being read before and after addition of the same amount of alkali as used in the determination.

$$c = p_1 - p_2$$

---

[25] The alkali must be made gas-free and kept in properly protected reservoirs. 25 to 30 ml. of 1 N NaOH are deaerated in the extraction chamber and transferred under oil to a reservoir made from a calcium chloride tube (Fig. 191). This will keep for a day. For larger amounts of solution, the apparatus shown in Fig. 192 is suggested. It consists of two leveling bulbs connected by a rubber tube 1 meter long. One of the bulbs (illustrated) is provided with a glass capillary and stopcock. The bulb is half-filled with absorbent solution and extracted by evacuation and shaking. Solution is delivered into the extraction chamber directly from the capillary of this reservoir.

To minimize error, all solutions must be measured very accurately, so that S varies within ± 0.05 ml. Also, sufficient time must elapse for temperature equilibrium to be established. (For other sources of error and refinements of technique, see the original paper.[26])

CALCULATION. See p. 714.

Determination of Plasma CO_2 Capacity. CO_2 capacity, as a clinical measure of acidosis, may be determined by the volumetric method of Van Slyke and Cullen (p. 693). For the manometric determination, admit a drop of caprylic alcohol into the capillary of the cup, followed by 1.5 ml. of 0.1 N lactic acid. One ml. of plasma (previously saturated with alveolar air) is introduced below the acid. The solutions are admitted to the extraction chamber, cock *b* sealed, the chamber evacuated and shaken 2 minutes. Then p_1 is read as described above with the gas volume at 2 ml. The gas is ejected, the ejected portion of the solution returned to the chamber, and the pressure reduced so that the gas space is again 2 ml. The value p_2 and the temperature are then read.

CALCULATION. Pressure of extracted air and $CO_2 = p_1 - p_2$.

The CO_2 capacity is obtained from this pressure by the use of the nomogram (Fig. 193).

Determination of Oxygen in Blood.[27] Oxygen may be determined manometrically on 1-ml. samples of blood with a variation within 0.2 volume per cent.

The special O_2 reagent[28] is emulsified by rotating, and 7.5 ml. are measured into the apparatus and deaerated by shaking in vacuo for three minutes. During the shaking, the mercury should be well in the neck below the bulb, to afford minimum exposure of surface, as mercury reacts with ferricyanide. One ml. of the well-mixed blood is drawn into a pipet, preferably such as described on p. 711. 6 ml. of the extracted reagent are forced into the cup, leaving 1.5 ml. in the chamber. The blood is introduced under the reagent directly into the chamber as described under "Measuring Samples." The pipet is carefully withdrawn and 1 ml. of reagent is permitted to flow into the chamber, rinsing through the blood in the capillary. The cock is sealed with a drop of mercury and the excess reagent discarded. The apparatus is evacuated and shaken for 3 minutes. 1 ml. of air-free 1 N NaOH is placed in the cup and the CO_2 absorbed by admitting 0.5 ml. of the hydroxide into the chamber under diminished pressure as described in CO_2 determination on whole blood.

The solution meniscus is brought to 2 ml. (*a*) and p_1 (pressure of $O_2 + N_2$) is read. (For low O_2 values it is preferable to read p_1 with *a* at 0.5 ml.) As in the volumetric method, O_2 may now be determined either by making a correction for N_2, or directly by absorption. In the first case, after p_1 has been

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[26] Austin, *J. Biol. Chem.*, **61**, 345 (1924), has described a modification of this procedure to be used in CO_2 analyses of serum obtained after ether anesthesia.

[27] Sendroy, *J. Biol. Chem.*, **91**, 307 (1931), describes a shorter method for hemoglobin by manometric O_2-capacity determination.

[28] *Special Oxygen Reagent.*

Potassium ferricyanide	3.0 g.
Saponin (Merck)	3.0 g.
Caprylic alcohol	3.0 ml.
Water to	1000 ml.

Low results for oxygen may be accounted for by saponin of low hemolytic activity, in which case the amount may be increased.

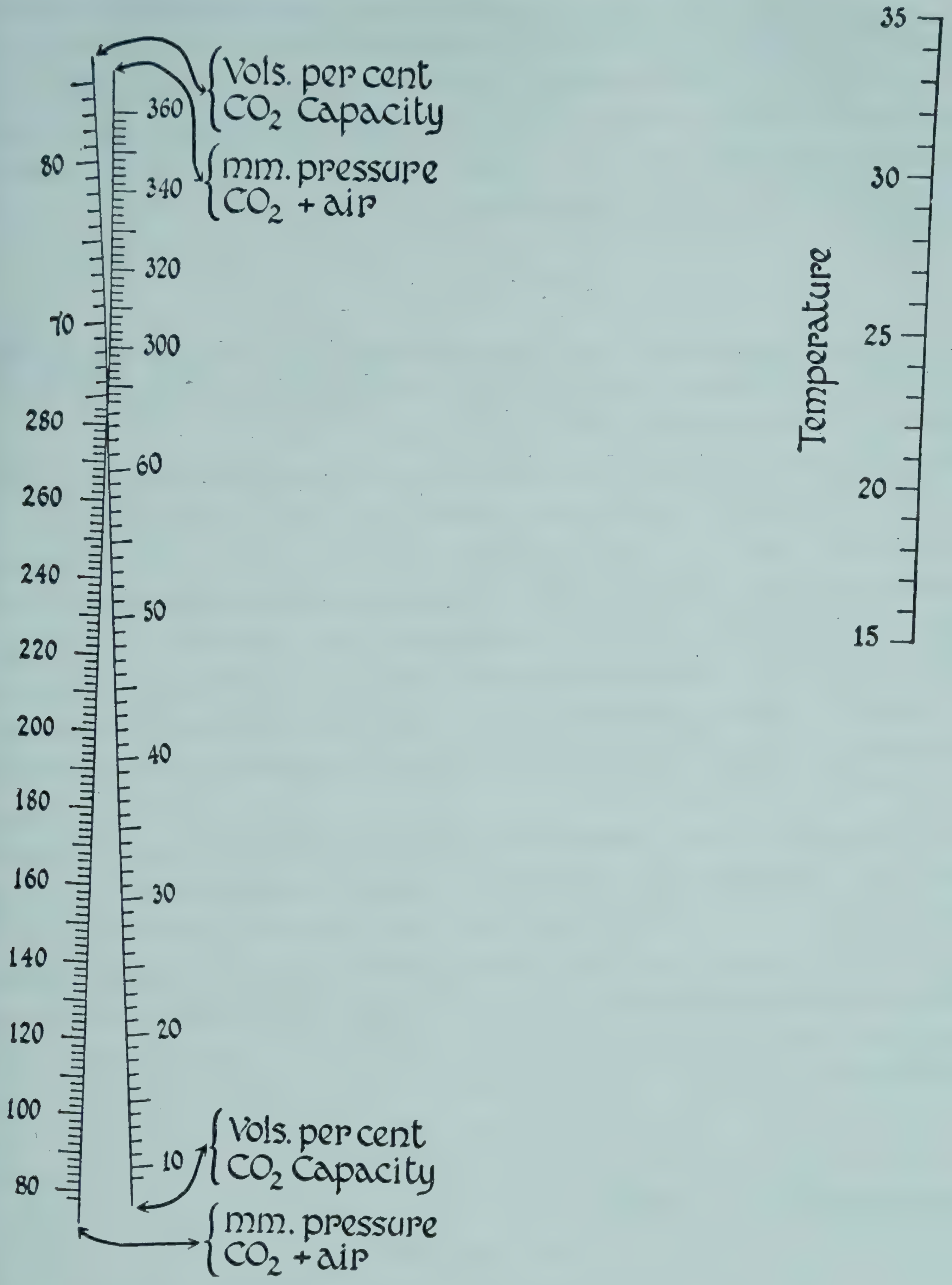

FIG. 193. NOMOGRAM FOR CALCULATION OF PLASMA CO_2 CAPACITY.

A straight line connecting the observed points on the pressure and temperature scales cuts the CO_2 capacity scale at the point indicating the capacity.

read, the gases are ejected from the chamber and p_2 is determined with the solution meniscus at the same *a* mark.

The absorption method is resorted to for greater precision or when other gases (e.g., CO) are present. After p_1 has been read, cock *e* is opened, with the leveling bulb in position to produce a gas space of 4 to 5 ml. Cock *e* is then closed. Next 1.5 ml. of hydrosulfite solution[29] is introduced into the cup

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[29] Grind up 100 g. of sodium hydrosulfite and 10 g. of sodium anthraquinone-β-sulfonate (Eastman Kodak Co.) and keep in stoppered bottle. To prepare absorbent solution stir

and by turning cock *b* admitted a drop at a time. As each drop trickles down, it absorbs O_2 and the mercury in the manometer falls. After a few drops have been admitted no further perceptible fall occurs. Cock *e* is then opened and the solution permitted to rise as near the stopcock as it will, with the leveling bulb in the lower ring. The remainder of 1 ml. of hydrosulfite is then added, completing the absorption of traces of O_2. The gas is brought to the same *a* volume and p_2 is read on the manometer.

$$p_{O_2} = p_1 - p_2 - c_{O_2}$$

To check the completeness of O_2 absorption and the absence of air leakage, expel the N_2 and measure p_3 in the gas-free apparatus. The N_2 should approximate 1.2 volumes per cent in the O_2 content determination, or 1.4 volumes per cent in O_2 capacity.

An alternative absorbent for O_2 is the alkaline pyrogallate reagent (p. 708). This is not so clean or rapid as the hydrosulfite, but is more stable and its use is advised when O_2 determinations are done only occasionally. The gummy precipitate formed with the blood mixture necessitates mixing the solutions in the bulb of the chamber, well below the *a* mark, resulting in a greater negative pressure for the admission of the absorbent. One ml. of alkaline pyrogallate is admitted dropwise over 4 or 5 minutes. The pressure is then allowed to rise to atmospheric so that the upper tube is rinsed by the solution, thus avoiding a reduction of vapor tension due to the strong alkali. Then p_2 is read. The apparatus is cleaned with water after using this absorbent.

The *c* correction, when oxygen is determined by absorption, is due to two factors, the lowering of the mercury meniscus by the absorbent and the trace of O_2 left in the reagent after one extraction. To determine *c*, deaerate 7.5 ml. of the special O_2 reagent for 3 minutes, expel 5 ml., leaving 2.5 in the chamber. Shake again for 3 minutes and then admit 1.5 ml. of air-free 1 N NaOH in the manner described for CO_2. Read p_1 with *a* at 0.5 and 2.0 ml. Then run in the absorbent as in the analysis and obtain p_2 at both points. Use c_{O_2} corresponding to *a* in the analysis.

$$c_{O_2} = p_1 - p_2$$

Eject residual N_2 and read p_3.

$$c_{N_2} = p_2 - p_3$$

If O_2 is determined indirectly by correcting for N_2 instead of by absorption, *c* is obtained in the same way, except that p_2 is measured after ejection of the $O_2 + N_2$, without preliminary absorption.

CALCULATIONS. The estimated corrections for dissolved O_2 and N_2 of blood in calculation of total or combined oxygen or oxygen capacity, given in the accompanying table, may vary somewhat with the cell content.

The corrections are subtracted from the volume per cent of gas as determined by the use of the table on p. 712.

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10 g. of this mixture in 50 ml. of 1 N KOH and quickly filter through cotton. Deaerate the solution and transfer to container under oil as described in the footnote on p. 715. One drop of 10 per cent $FeCl_3$ still further accelerates the absorption activity. The solubility of N_2 in this medium is negligible.

ESTIMATED CORRECTIONS FOR DISSOLVED O_2 AND N_2 IN BLOOD

Blood	*Determined*	*Sought*	*Correction to Subtract*	
			Vol. per cent	*M. per l.*
Venous	Total O_2	Combined O_2	0.1 (O_2)	0.04 (O_2)
Arterial	Total O_2	Combined O_2	0.2 (O_2)	0.09 (O_2)
Saturated with air at 20°, 760 mm.	Total O_2	Combined O_2 (O_2 capacity)	0.5 (O_2)	0.22 (O_2)
Venous	Total $O_2 + N_2$	Combined O_2	1.3 ($O_2 + N_2$)	0.57 ($O_2 + N_2$)
Arterial	Total $O_2 + N_2$	Combined O_2	1.5 ($O_2 + N_2$)	0.62 ($O_2 + N_2$)
Saturated with air at 20°, 760 mm.	Total $O_2 + N_2$	Combined O_2 (O_2 capacity)	1.9 ($O_2 + N_2$)	0.85 ($O_2 + N_2$)
Venous	Total $O_2 + N_2$ or $CO + O_2 + N_2$	Total O_2 or $CO + O_2$	1.2 (N_2)	0.53 (N_2)
Arterial	Total $O_2 + N_2$ or $CO + O_2 + N_2$	Total O_2 or $CO + O_2$	1.2 (N_2)	0.53 (N_2)

Determination of Carbon Monoxide Hemoglobin, of Methemoglobin, and of Hemoglobin by the Carbon Monoxide Capacity Method:[30] ***Principle.*** Carbon monoxide is liberated from combination with hemoglobin by treatment with an acid ferricyanide solution. CO_2 and O_2 are absorbed with alkaline pyrogallol solution and CO determined by correcting for N_2 in the residual gas.

For determination of hemoglobin the blood is saturated with CO and the CO capacity determined. Volumes of CO absorbed are identical with those for O_2. This gives active hemoglobin.

CO without the aid of hydrosulfite does not change methemoglobin to carboxyhemoglobin. The difference between CO capacity with and without hydrosulfite treatment represents methemoglobin. Hemochromogen behaves like methemoglobin but is rarely present.[31]

Determination of Hemoglobin** (**Active**) **by CO Capacity. **Draw 1 drop of caprylic alcohol into the capillary beneath the cup of the manometric apparatus. Measure 4.75 ml. of water into the cup. With stopcock pipet provided with rubber tip (see p. 711), run 2 ml. of blood directly into the chamber, followed by the 4.75 ml. of water. Place 1 to 2 ml. of mercury in the cup above the chamber. Fill the outlet capillary of a Hempel gas pipet containing CO (see Fig. 194) with mercury. Fit the tip of the pipet into the bottom of the cup as shown in the illustration. Turn the two cocks shown in the illustration so that CO gas[33] can flow from the pipet into the chamber. The flow is regu-**

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[30] Van Slyke and Hiller: *J. Biol. Chem.*, **78**, 807 (1928); **84**, 205 (1929). Methods for smaller amounts of blood are also given. For a somewhat more accurate procedure for the determination of carbon monoxide in blood, see Sendroy and Liu: *J. Biol. Chem.*, **89**, 133 (1930).

[31] Conant, Scott, and Douglass, *J. Biol. Chem.*, **76**, 223 (1928), give a method suitable in the presence of hemochromogen.

[32] The bulbs are of about 50-ml. capacity each. The capillary is of 1-mm. bore. When the pipet is not in use, a little mercury is let into the capillary leading to the CO bulb, to seal the three-way cock and prevent leakage around it. This drop of mercury in the capillary to the right of the cock is shown in the illustration. This pipet may also be used to store air-free solutions. In this case the solution replaces the CO gas shown above, and mercury is used where water is indicated in the above figure. Supplied by the makers of the Van Slyke-Neill apparatus.

[33] *CO Gas.* Connect the lower openings of two 5-liter aspirator bottles with rubber tubing at least 15 mm. wide. Fill one (*A*) completely with water. *A* is also fitted with a thistle tube with stopcock and a side tube with stopcock connecting with a large test tube carrying a safety thistle tube containing mercury passing through the stopper only and a

lated by the cock leading to the mercury leveling bulb of the manometric apparatus. With the leveling bulb in mid-position (a little below the bottom of the chamber), open this cock slowly, withdrawing mercury from the chamber until CO enters to the 2-ml. mark. Close the cock of the chamber, seal with a drop of mercury, and evacuate. With the mercury meniscus at the 50-ml. mark, shake until equilibrium is reached (1 minute or longer as determined in blank below). Eject the mixture of gases from the chamber.

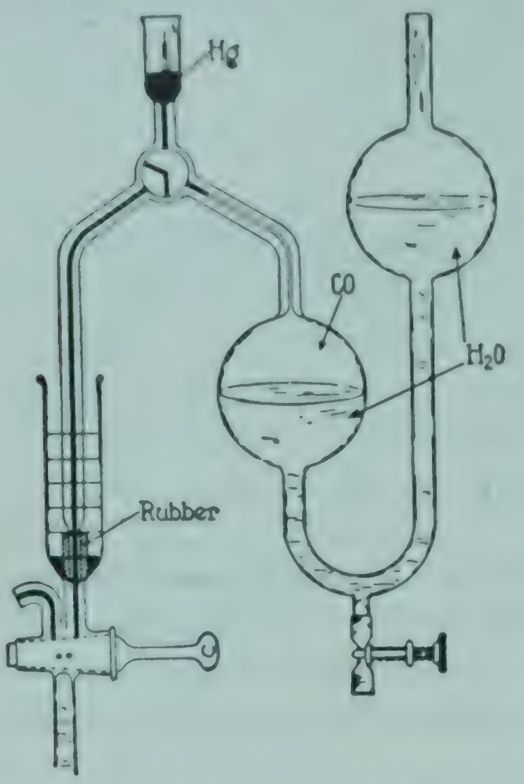

FIG. 194. HEMPEL PIPET PROVIDED WITH THREE-WAY STOPCOCK[32] (VAN SLYKE AND HILLER).

Evacuate until blood solution is in lower fourth of chamber. Put about 1 ml. of mercury and 2 to 3 ml. of water in the cup. Through the mercury seal introduce 0.25 ml. of acid ferricyanide[34] solution, using a rubber-tipped buret made by fusing a stopcock onto a pipet graduated in 0.01-ml. divisions. Before the tip of the buret is inserted into the mercury, move it through the water layer to dislodge ferricyanide crystals or air bubbles. Fill the capillary and bore of the cock with mercury but run none into the chamber. Evacuate, lowering the mercury to the 50-ml. mark. Shake slowly for about 5 seconds, and then vigorously for 3 minutes. Admit mercury until gas space is reduced to 5 to 6 ml. Measure 2 ml. of air-free 1 N NaOH[34] into the cup, letting it run in slowly with the tip against the bottom of the cup. Allow 1 ml. of the NaOH to flow slowly into the chamber. Absorption of CO_2 is complete in less than 1 minute. Bring the volume of gas to 2 ml. Record the manometer reading as p_1. Eject the gas. Seal the stopcock with a drop of mercury. Lower the fluid meniscus to 2 ml. Read the manometer again (p_2).

CALCULATION. The hemoglobin content of the blood in terms of CO- or O_2-combining power is calculated by the equation:

$$\text{CO or O}_2\text{ capacity} = (p_1 - p_2 - c)f$$

where f is a factor obtained from the table on p. 712 and c is determined by a blank analysis in which the procedure described above is repeated in every detail except that 2 ml. of water are substituted for the 2 ml. of blood. The correction is calculated as $c = p_1 - p_2$. The value of c, once accurately determined, can be used as a constant since it is small and insensitive to ordinary changes in laboratory conditions.

Determination of Total Hemoglobin and Methemoglobin. **Wash apparatus with three successive portions of 10 to 15 ml. of water, to the first of which**

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second thistle tube, with stopcock, reaching to the bottom of the test tube. Run into the test tube 3 ml. of anhydrous formic acid, and then, slowly, concentrated H_2SO_4, gently warming the mixture with a microburner. When about 300 ml. of CO and air have collected in *A*, drive this out by opening the thistle tube and lifting *B*. Continue generation until the CO from all the formic acid is collected in *A*. Clamp the tube between the two bottles with a screw clamp and detach the test tube. *Carry out the entire procedure in a hood or in a free draft of air.* Smaller vessels may be used if less CO is desired.

[34] *Acid Ferricyanide Solution.* To 92 volumes of stock solution, containing 32 g. of $K_3Fe(CN)_6$ per 100 ml., add 8 volumes of concentrated lactic acid of sp. gr. 1.2. It may be used for more than 2 months. *1 N NaOH.* Rendered air-free as described on p. 715.

a little hydrosulfite solution is added. This is done by evacuating to the 50-ml. mark, running in the water, shaking for 15 to 20 seconds, and ejecting the solution.

Draw 2 drops of caprylic alcohol into the capillary beneath the cup. Measure into the cup 4.3 ml. of water. Using the stopcock pipet with rubber tip, run 2 ml. of blood directly into the chamber, followed by a few drops of the water in the cup to wash blood through the capillary. From a microburet (see above) run 0.4 ml. of the ammoniacal sodium hydrosulfite[35] solution into the chamber, followed by the water remaining in the cup. Put 1 to 2 ml. of mercury in the cup. Lower the mercury in the chamber to the 50-ml. mark. Run in CO from the Hempel pipet until the pressure on the manometer rises about 150 mm., the mercury remaining at the 50-ml. mark. If 100 mm. of CO are run in and then mercury admitted from the leveling bulb to raise the level to the 50-ml. mark, the proper pressure will usually be attained. Shake for 1½ minutes. Eject gases from the chamber. Determine CO exactly as in the method above for active hemoglobin, except that 0.30 ml. instead of 0.25 ml. of the ferricyanide solution is run in. Determine correction *c* by running a blank test on 2 ml. of water (see above). This *c* will be somewhat greater than in the method for active hemoglobin. The calculation is otherwise the same and gives total hemoglobin.

Methemoglobin = total hemoglobin − active hemoglobin

Manometric Determination of Other Substances. See footnote 22, p. 709. Other manometric methods are those for urea using the urease[36] or hypobromite[37] procedures, oxalic acid or calcium,[38] total reducing substances,[39] and fermentable sugars[40] in blood and urine, lactic acid in blood,[41] potassium in serum,[42] gas in fermentation,[43] primary amino nitrogen,[44] cysteine and cystine,[45] gas mixtures,[46] carbon in organic substances,[47] chloride in serum and urine,[48] phosphorus in organic compounds,[49] carboxyl groups in amino acids,[50] inorganic sulfate,[51] magnesium,[52] amino acids in blood,[53] amino acids in urine,[54] arginase,[55] total carbon and its radioactivity.[56] For some of these methods, later references will be found in Chapters 23 and 31.

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[35] *Ammoniacal Sodium Hydrosulfite.* Pour 50 ml. of diluted (1:50) ammonia solution upon 2.0 g. of pulverized $Na_2S_2O_4$ contained in a 100-ml. beaker. Cover at once with a layer of paraffin oil. Dissolve by stirring with a rod for a few seconds.

[36] Van Slyke: *J. Biol. Chem.*, **73**, 695 (1927).

[37] Van Slyke: *J. Biol. Chem.*, **83**, 449 (1929).

[38] Van Slyke and Sendroy: *J. Biol. Chem.*, **84**, 217 (1929).

[39] Van Slyke and Hawkins: *J. Biol. Chem.*, **79**, 739 (1928).

[40] Van Slyke and Hawkins: *J. Biol. Chem.*, **83**, 51 (1929).

[41] Hastings and Avery: *J. Biol. Chem.*, **94**, 273 (1931).

[42] Kramer and Gittleman: *Proc. Soc. Exptl. Biol. Med.*, **24**, 241 (1926).

[43] Raymond: *J. Biol. Chem.*, **83**, 611 (1929).

[44] Van Slyke: *J. Biol. Chem.*, **83**, 425 (1929).

[45] Baernstein: *J. Biol. Chem.*, **89**, 125 (1930).

[46] Van Slyke and Hanke: *J. Biol. Chem.*, **95**, 569, 587, 599 (1932).

[47] Van Slyke, Page, Irvine, and Kirk: *J. Biol. Chem.*, **100**, xciii (1933).

[48] Sendroy: *J. Biol. Chem.*, **109**, lxxxi (1935).

[49] Kirk: *J. Biol. Chem.*, **106**, 191 (1934).

[50] Van Slyke, Dillon, MacFayden, and Hamilton: *J. Biol. Chem.*, **141**, 627, 671 (1941).

[51] Hoagland: *J. Biol. Chem.*, **136**, 543 (1940).

[52] Hoagland: *J. Biol. Chem.*, **136**, 553 (1940).

[53] Hamilton and Van Slyke: *J. Biol. Chem.*, **150**, 231 (1943).

[54] Van Slyke, MacFayden, and Hamilton: *J. Biol. Chem.*, **150**, 251 (1943).

[55] Van Slyke and Archibald: *J. Biol. Chem.*, **165**, 293 (1946).

[56] Van Slyke, Steele, and Plazin: *J. Biol. Chem.*, **192**, 769 (1952).

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25

Energy Metabolism

Historical. Combustion in living beings was first described by Lavoisier (1743–1794). This brilliant chemist discovered the true nature of oxygen, and how its union in the body with carbon and hydrogen resulted in the formation of carbon dioxide and water and in the production of heat. In experiments on man he determined that oxidation was increased by food, by a cold environment, and by muscular work. He knew the main constituents of the atmosphere and that nitrogen played no active part in animal respiration. Lavoisier was under the false impression that oxygen decomposed some fluid in the lung, causing the liberation of hydrogen and carbon with the subsequent oxidation of these elements and their excretion as carbon dioxide and water in the expired air. Scientists of a later date abandoned the theory of oxidation in the lungs, and favored the blood as the site of these chemical changes. This belief was strengthened by the discovery of gases in the blood by Magnus in 1837. Later it became known that, for the most part, oxidation occurs in the tissues and that the blood simply carries the gases to and from the lungs.

In 1842 Liebig announced that the substances burned in the body were carbohydrate, fat, and protein, and suggested that the urinary nitrogen would serve as an index of the extent of protein destruction. Ten years later Bidder and Schmidt described protein metabolism, and shortly afterwards Carl Voit measured protein waste by determining the amount of nitrogen in the excreta. Voit made the important discovery that muscular exercise did not increase protein metabolism. During many years of careful work, Rubner, a pupil of Voit, determined the fuel values of the foodstuffs and by means of a respiration calorimeter showed that the heat production as calculated from the respiratory exchange was the same as that obtained by the direct measurement of the heat given off by the body. In 1883, Rubner demonstrated the relationship between the surface area of the body and the heat production, thus providing a basis for comparison of the metabolism of different individuals.

In 1915 DuBois devised the most satisfactory method for estimating the surface area of the body, and later published normal standards of heat production for males and females, the accuracy of which has been fairly well established.

In America, Atwater, Armsby, Benedict, Lusk, DuBois, and their many associates have added valuable contributions to the knowledge of normal metabolism. Benedict and those associated with him in the Carnegie Nutrition Laboratory have in addition perfected various forms of respiration apparatus, and they are chiefly responsible for the extensive use of calorimetry in the clinic.

Physical and Chemical Bases of Animal Calorimetry.

GASES. Gases are compressible fluids. The molecules of which they are composed are in constant motion and tend to disperse. The gas therefore expands and fills all parts of the containing vessel and exerts pressure upon the enclosing walls. Heat increases the tendency to expand and so increases the pressure unless the container permits the gas to assume a larger volume. If the temperature remains constant and pressure is applied to the container, the molecules are forced closer together and the volume is decreased. Therefore, when we speak of a volume of gas, we must also state the temperature and pressure under which it was measured. Regardless of the experimental conditions of measurement, gas volumes are always recorded as the volume at standard temperature (0° Centigrade or 273° Absolute) and standard pressure (760 mm. of mercury at 0° C.[1]). This is sometimes referred to as *standard conditions* or as N.T.P. (normal temperature and pressure).

If the temperature is kept constant, the volume of a gas varies inversely with the pressure (*Boyle's Law*). Therefore, to express a volume of gas, measured at some other pressure, as its volume at 760 mm., the formula is:

$$\text{Volume} \times \frac{\text{Pressure (corrected)}}{760} = \text{Volume at 760 mm.}$$

If the pressure on a gas remains the same and the temperature changes, the volume of the gas will vary directly $^{1}/_{273}$ (or 0.00367) of its volume at 0° C. for each degree of change in temperature (*Charles' or Gay Lussac's Law*). The coefficient of expansion of nearly all gases is 0.00367.

To convert the volume of a gas at temperature t° C., and at constant pressure, to its volume at 0° C., the formula is:

$$\text{Volume} \times \frac{273}{273 + t^\circ\ \text{C.}} = \text{Volume at } 0^\circ\ \text{C.}$$

The two conversion equations given may be combined into a single equation as follows:

$$\frac{P \times V}{273 + t} = \frac{760 \times V_0}{273}$$

where P and V represent the experimentally determined pressure and volume, corrected if necessary, at the temperature t° C., and V_0 represents the volume under standard conditions.

If a volume of gas is in contact with water, evaporation continues until the tendency of the water to evaporate is equal to the tendency of its vapor to condense, at which time the gas is said to be saturated with water vapor. The total pressure of the saturated gas and water vapor is equal to the sum of the pressures of the gas and of the water vapor (*Dalton's Law*). These are spoken of as *partial pressures.* The partial pressure of the water vapor increases with temperature. To determine the pressure of the gas in a dry state, the partial pressure of water vapor at the given temperature (see p. 709) must be deducted from the barometric pressure.

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[1] The reading on a brass-scale barometer is correct for only one temperature and must be corrected by the use of a correction table which gives the height at which the column of mercury would stand if the temperature were 0° C. For such a table, refer to a handbook of chemistry or physics. See also p. 697.

Example. The expired air of a man has a volume of 100 liters, the temperature in the gasometer is 22° C., and the brass scale barometer reads 767 mm. The pressure of expired air, *dry*, is:

$$767 \text{ mm.} - \underset{\textit{Barometric correction}}{2.72} - \underset{\textit{Water vapor}}{19.63} = 744.65 \text{ mm.}$$

The volume of dry air at 0° C. and 760 mm. is:

$$100 \times \frac{744.65}{760} \times \frac{273}{273 + 22} = 90.66 \text{ liters}$$

Equal volumes of different gases or vapors under like conditions of temperature and pressure contain the same number of molecules (*Avogadro's Law*). Thus the density or *mass per volume* of any gas or vapor will depend upon the weight of its molecule. The molecular weight in grams of any gas or vapor has a volume of practically 22.4 liters at 0° C. and 760 mm. Hence the density of 1 liter of a gas at 0° C. and 760 mm. is molecular weight ÷ 22.4.

Density of Gases at 0° C. and 760 mm.

1 liter of oxygen weighs 1.4292 g.

$$1 \text{ g. of oxygen occupies } \frac{1}{1.4292} = 0.6997 \text{ liter}$$

1 liter of carbon dioxide weighs 1.9652 g.

$$1 \text{ g. of carbon dioxide occupies } \frac{1}{1.9652} = 0.5089 \text{ liter}$$

HEAT. The heat which we recognize by *temperature* is the energy of molecular motion. This property is imparted to matter by chemical action, electric currents, and mechanical work. In animals the chemical source only need be considered and we may confine our attention to that form of combustion in which the substance finally appears in the completely oxidized form.

In animal calorimetry the unit of heat is the *large Calorie*, abbreviated Cal., which is defined as the amount of heat necessary to raise the temperature of 1 liter of water from 15° C. to 16° C.

Heat of Combustion in a Calorimeter.

1 g. of *hydrogen* gas produces 34.5 Cal.
1 g. of *charcoal* produces 8.0 Cal.
1 g. of *starch* produces about 4.2 Cal.
1 g. of *glucose* produces about 3.74 Cal.
1 g. of *sucrose* produces about 3.96 Cal,

Heat of Combustion in Animals.

1 g. of average *carbohydrate* produces about 4 Cal.
1 g. of average *fat* produces about 9 Cal.
1 g. of average *protein* produces about 4 Cal.

Oxidation of Carbohydrates. Carbohydrates, by the processes of digestion, undergo hydrolytic cleavage if necessary and are absorbed through the intestinal wall into the blood mainly in the form of glucose. Under normal conditions the blood contains 0.1 per cent or less of this sugar in the free state; over 300 g. or more may be stored as glycogen in the body tissues, principally in the muscles and liver. Glycogen is readily reconverted to glucose and it serves as a deposit to be drawn upon in an emergency. Glucose is the favorite body fuel, and is used prodigally when the supply is plentiful and thriftily in periods of starvation.

The oxidation of glucose is represented as follows:

	$C_6H_{12}O_6$ +	$6O_2$	= $6CO_2$ +	$6H_2O$
Molecular weights in grams..........	180	192	264	108
Heat and volume equivalents........	673.2 Cal.	134.34 liters	134.34 liters	
Heat and volume equivalents........	5.011 Cal. per 1 liter		1 liter	

Carbohydrates contain hydrogen and oxygen in the same proportion as is found in water. When these substances burn, outside oxygen is used to unite with the carbon, forming a volume of carbon dioxide equal to the volume of oxygen absorbed. The ratio of the volume of carbon dioxide produced to the volume of oxygen absorbed is known as the *respiratory quotient* (R.Q.). This has a different value for each of the major food components and serves to determine what substances are being burned. From the above equation it is seen that the respiratory quotient for glucose is $\frac{CO_2}{O_2} = 1.0$ and that 1 liter of oxygen represents a liberation of 5.011 Cal. In animal calorimetry the heat equivalent of 1 liter of oxygen is generally accepted as 5.047 Cal. when carbohydrates are burned in the body.

Oxidation of Fats. The fats and oils of our food are largely mixtures of palmitin, stearin, and olein (see Chapter 3). These substances are of similar chemical composition and when they undergo oxidation they yield about the same amount of heat. After absorption, fat passes from the blood to the tissues, where it is either burned or stored for future use. Fats have a high fuel value and they are continually being used by the body for this purpose. In the absence of carbohydrates, fats supply over 80 per cent of the body heat.

Fat combustion is usually represented by the oxidation of palmitin as follows:

	$2(C_{51}H_{98}O_6)$ +	$145O_2$	= $102CO_2$ +	$98H_2O$
Molecular weight in grams........	1612	4640	4488	1764
Heat and volume equivalents.....	15233.4 Cal.	3246.6 liters	2283.9 liters	
Heat and volume equivalents.....	4.655 Cal. per 1 liter		0.704 liter	

From the above equation it is seen that when palmitin is burned the respiratory quotient is 0.704 and that 1 liter of absorbed oxygen liberates

ANALYSIS OF THE OXIDATION OF MIXTURES OF CARBOHYDRATE AND FAT*
(Table of Zuntz and Schumburg modified by Lusk)

R.Q.	*Percentage of Total Oxygen Consumed*		*Percentage of Total Heat Produced*		*Cal. per liter of* O_2		
	Carbo-hydrate	*Fat*	*Carbo-hydrate*	*Fat*	*Number*	*Log*	*Log + log 60*
0.707	0	100.00	0	100.00	4.686	.67080	.44895
.71	1.02	99.00	1.10	98.90	4.690	.67114	.44929
.72	4.44	95.60	4.76	95.20	4.702	.67228	.45043
.73	7.85	92.20	8.40	91.60	4.714	.67342	.45157
.74	11.30	88.70	12.00	88.00	4.727	.67456	.45271
.75	14.70	85.30	15.60	84.40	4.739	.67569	.45384
.76	18.10	81.90	19.20	80.80	4.751	.67682	.45497
.77	21.50	78.50	22.80	77.20	4.764	.67794	.45609
.78	24.90	75.10	26.30	73.70	4.776	.67906	.45721
.79	28.30	71.70	29.90	70.10	4.788	.68018	.45833
.80	31.70	68.30	33.40	66.60	4.801	.68129	.45944
.81	35.20	64.80	36.90	63.10	4.813	.68241	.46056
.82	38.60	61.40	40.30	59.70	4.825	.68352	.46167
.83	42.00	58.00	43.80	56.20	4.838	.68463	.46278
.84	45.40	54.60	47.20	52.80	4.850	.68573	.46388
.85	48.80	51.20	50.70	49.30	4.862	.68683	.46498
.86	52.20	47.80	54.10	45.90	4.875	.68793	.46608
.87	55.60	44.40	57.50	42.50	4.887	.68903	.46718
.88	59.00	41.00	60.80	39.20	4.899	.69012	.46827
.89	62.50	37.50	64.20	35.80	4.911	.69121	.46936
.90	65.90	34.10	67.50	32.50	4.924	.69230	.47045
.91	69.30	30.70	70.80	29.20	4.936	.69339	.47154
.92	72.70	27.30	74.10	25.90	4.948	.69447	.47262
.93	76.10	23.90	77.40	22.60	4.961	.69555	.47370
.94	79.50	20.50	80.70	19.30	4.973	.69663	.47478
.95	82.90	17.10	84.00	16.00	4.985	.69770	.47585
.96	86.30	13.70	87.20	12.80	4.998	.69877	.47692
.97	89.80	10.20	90.40	9.58	5.010	.69984	.47799
.98	93.20	6.83	93.60	6.37	5.022	.70091	.47906
.99	96.60	3.41	96.80	3.18	5.035	.70197	.48012
1.00	100.00	0	100.00	0	5.047	.70303	.48118

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* The last column has been added to facilitate the expression of oxygen absorbed per minute in terms of Calories per hour. Characteristics are omitted (see last paragraph, p. 741).

4.655 Cal. Zuntz and Schumburg obtained slightly different figures for the oxidation of fat in the body. In their work the respiratory quotient was found to be 0.707 and the heat value for 1 liter of oxygen 4.686 Cal. If only mixtures of carbohydrate and fat were oxidized, the respiratory quotients would vary between 0.707 and 1.00, and from their value it should be possible to determine the proportions of each of these foodstuffs burned. A table analyzing the oxidation of such mixtures on the basis of the so-called *nonprotein respiratory quotient* was prepared by Zuntz and Schumburg and modified by Lusk (see p. 727).

Protein Metabolism. Proteins in the diet are completely broken down in the gastrointestinal tract to the form of amino acids, in which form they are absorbed into the blood and pass to the various parts of the body. Here they may be utilized either for incorporation into new tissue protein which is continually being broken down and resynthesized, or they may undergo metabolic reactions leading to the formation of nonprotein nitrogenous compounds of importance to the body, such as creatine, thyroxine, adrenaline, etc. Presumably they may also be utilized for the direct production of energy, although under ordinary circumstances protein is not considered to be primarily a fuel. Regardless of the intermediate steps in protein metabolism, which are discussed in detail in Chapter 33, the end products of protein breakdown in the animal body include not only carbon dioxide and water, as for carbohydrates and fats, but also the urea of the urine and certain other nitrogenous constituents of the urine and feces, as well as such compounds as urinary sulfate produced by the oxidation of sulfur-containing amino acids. Hence to evaluate the contribution of protein to the total metabolism, and to distinguish it from the nonprotein metabolism, the analysis of urine and feces is necessary in addition to the measurement of the respiratory gas exchange.

A computation of protein metabolism by Loewy is as follows:

100 g. of meat protein contains:					
	52.38 g. C	7.27 g. H	22.68 g. O	16.65 g. N	1.02 g. S
of which there are eliminated in the urine:					
	9.406 g. C	2.663 g. H	14.099 g. O	16.28 g. N	1.02 g. S
in the feces:					
	1.471 g. C	0.212 g. H	0.889 g. O	0.37 g. N	
leaving a residuum for the respiratory process of:					
	41.5 g. C	4.4 g. H	7.69 g. O		
Deduct intramolecular water:					
		0.961 g. H	7.69 g. O		
leaving					
	41.5 g. C	3.439 g. H	for oxidation.		

When CO_2 is formed, 12 g. C unite with 32 g. O

$$\text{Therefore 41.5 g. C unite with } \frac{32 \times 41.5}{12} = 110.67 \text{ g. O}$$

When H_2O is formed, 2 g. H unite with 16 g. O

$$\text{Therefore 3.439 g. H unite with } \frac{16 \times 3.439}{2} = 27.512 \text{ g. O}$$

Total oxygen absorbed..138.18 g.
Total carbon dioxide produced..............41.5 g. C + 110.67 g. O = 152.17 g.

In the computation above, 1 g. of urinary nitrogen represents:

$$\frac{100 \text{ g. protein}}{16.28} = 6.15 \text{ g. of muscle protein}$$

$$\frac{138.18 \text{ g. } O_2}{16.28} = 8.49 \text{ g. of } O_2 \text{ absorbed}$$

$$\frac{152.17 \text{ g. } CO_2}{16.28} = 9.35 \text{ g. of } CO_2 \text{ produced}$$

6.15 g. protein × 4.25 (Cal.) = 26.14 Cal.
8.49 g. O_2 × 0.6997 (liters) = 5.94 liters of O_2 for protein
9.35 g. CO_2 × 0.5089 (liters) = 4.76 liters of CO_2 from protein

$$\text{Respiratory quotient for meat protein} = \frac{4.76}{5.94} = 0.801$$

Based on the analytical figures for the average protein it is estimated that 1 g. of urinary nitrogen represents the metabolism of 6.25 g. of protein, the absorption of 5.91 liters of oxygen, the production of 4.76 liters of carbon dioxide, and the liberation of 26.51 Cal.

An example of the practical application of the above *constants* is as follows:

A man (aged 38.5 yrs., height 159 cm., weight 90 lb.) was tested 14 hours after the last meal. An analysis of the urine showed an excretion of 0.16 g. of nitrogen per hour. The total respiratory exchange showed an absorption of 12.52 liters of oxygen and the production of 9.3 liters of carbon dioxide per hour.

0.16 g. of urinary nitrogen represents

0.16 × 6.25 = 1 g. of protein metabolized with the absorption of
0.16 × 5.91 = 0.95 liter of O_2, the production of
0.16 × 4.76 = 0.76 liter of CO_2, and the liberation of
0.16 × 26.51 = 4.24 Cal.

Total CO_2 (or O_2) − protein CO_2 (or O_2) = nonprotein CO_2 (or O_2)
9.3 liters CO_2 − 0.76 liter CO_2 = 8.54 liters CO_2
12.52 liters O_2 − 0.95 liter O_2 = 11.57 liters O_2

$$\frac{8.54 \text{ liters } CO_2}{11.57 \text{ liters } O_2} = 0.74 \text{ (nonprotein R.Q.)}$$

From Zuntz and Schumburg's table (modified by Lusk, see p. 727), it is seen that when the nonprotein R.Q. is 0.74, 1 liter of O_2 represents the liberation of 4.727 Cal., and 12 per cent of the nonprotein heat comes from carbohydrate and 88 per cent from fat.

11.57 liters O_2 × 4.727 = 54.69 Cal. (nonprotein)
12 per cent of 54.69 = 6.56 Cal. from carbohydrate
88 per cent of 54.69 = 48.13 Cal. from fat
54.69 + 4.24 (protein Cal.) = total of 58.93 Cal. per hour

In this computation 7 per cent of the body heat was derived from the combustion of protein, 11 per cent from carbohydrate, and 82 per cent from fat.

As Richardson points out, the measured respiratory quotient is not necessarily the metabolic quotient and gives no information concerning the intermediate steps in the conversion of consumed O_2 to CO_2. Nonoxidative processes which promote CO_2 production, such as acid formation (e.g., diabetes or excessive muscular activity) or acid retention (e.g., nephritis), and conditions which diminish the CO_2 output (e.g., insulin or alkali therapy, etc.) exercise an important influence on the respiratory quotient. Taking these factors into account, however, studies of conditions of abnormal carbohydrate and fat metabolism such as diabetes and ketosis, and of the physiology of food utilization and muscular activity, have been greatly advanced by measurements of the respiratory quotient.

Respiration. The ventilation of the lung and the exchange of gases between the alveolar air and the blood are usually referred to as external respiration, as contrasted with the exchange of gases in the tissues, known as internal respiration (see Chapter 12). Any disproportion between external and internal respiration affects the oxygen or carbon dioxide content of the blood. The tension of oxygen in the blood is lowered when the lungs are extensively disordered, as in pneumonia, and during periods of supreme muscular effort; under these conditions the oxygen requirement of the tissues is greater than the oxygenating power of the lung. An unusual increase in the supply of oxygen in the lung has but a very slight effect on the tension of oxygen in the blood, and practically no effect on the rate of combustion in the tissues. Of greater interest is the carbon dioxide tension of the blood, which is nicely regulated by an adjustment of the ventilation of the lung, and of the circulation of the blood, so that these mechanisms parallel the rate of combustion in the tissues.[2]

There is a general parallelism between the heat production, the heart rate, and the ventilation rate. However, the heart rate is not solely determined by the requirements of respiration, and ventilation can be voluntarily controlled. A rapid heart and an increased ventilation rate make one suspect that the metabolism is increased, but the rate of heat production cannot be predicted from these phenomena.

EXTERNAL RESPIRATION. Atmospheric air is of very constant composition the world over. Advantage is taken of this fact in computing the respiratory exchange, that is, the amount of oxygen absorbed and the amount of carbon dioxide produced in a given time.

Atmospheric air has the following composition:

Carbon dioxide	0.03 per cent
Oxygen	20.94 per cent
Nitrogen	79.03 per cent

Under ordinary conditions, at each breath a person inspires about 500 ml. of air and of this amount only 350 ml. actually enter the lung and diffuse more or less with the 3000 ml. already there. The expired air includes the 140 ml. of the "dead space" and 350 ml. of partially mixed air from the lung (see Fig. 195). Expired air contains about 16 per cent oxygen, 4 per cent carbon dioxide, and 80 per cent nitrogen.

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[2] For further discussion of the gas exchange in blood and tissues, see Chapter 24.

If we exhale forcibly and collect the last portion, the so-called alveolar air, we find that it contains about 14.5 per cent of oxygen and 5.6 per cent of carbon dioxide. That is, the air deep in the lung, where it comes in close proximity to the blood, has the greatest concentration of carbon dioxide and the lowest concentration of oxygen. These gases are in equilibrium with the gases in the arterial blood.

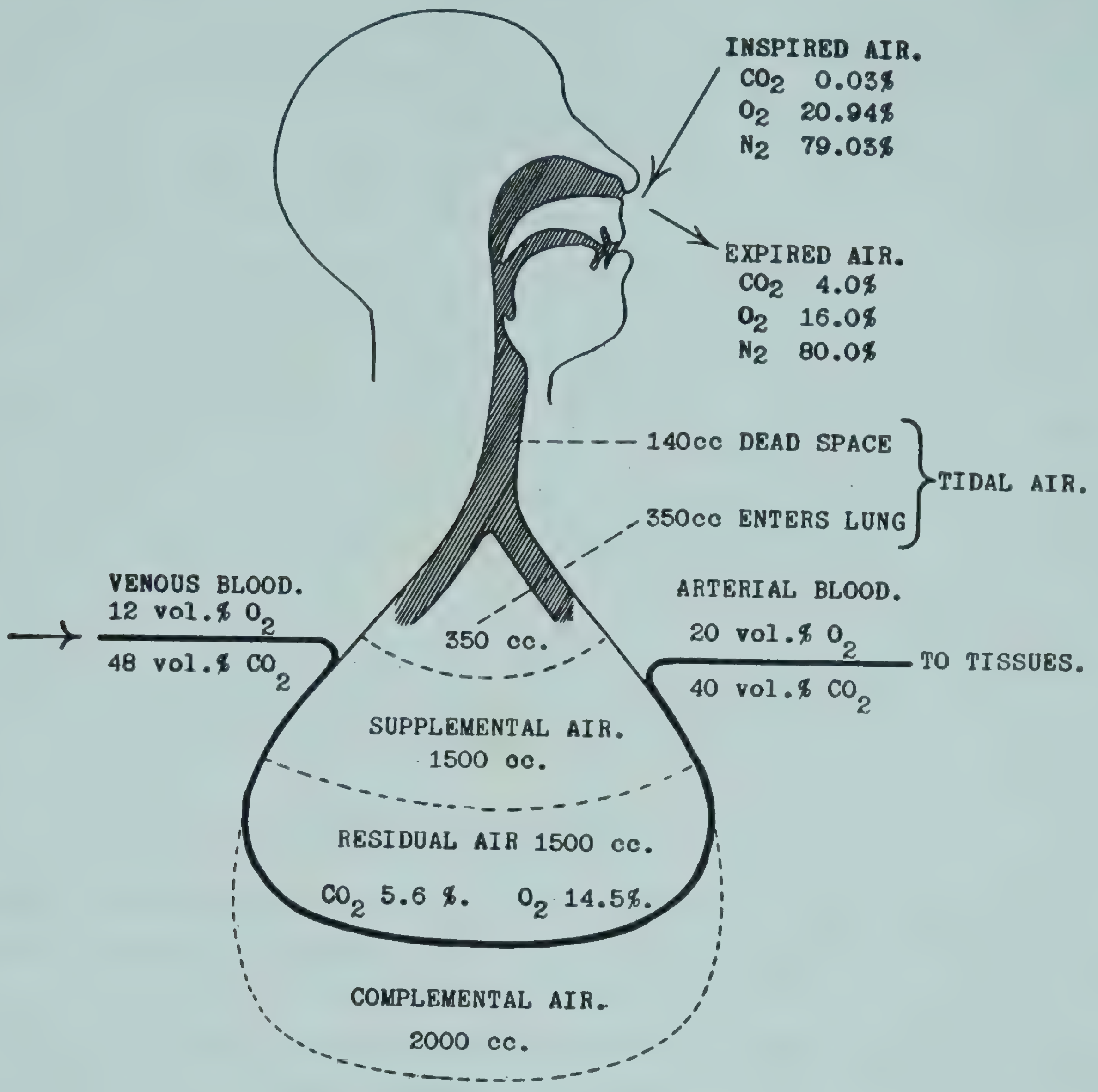
INSPIRED AIR.
CO_2 0.03%
O_2 20.94%
N_2 79.03%
EXPIRED AIR.
CO_2 4.0%
O_2 16.0%
N_2 80.0%
140cc DEAD SPACE
350cc ENTERS LUNG
TIDAL AIR.
VENOUS BLOOD.
12 vol.% O_2
48 vol.% CO_2
ARTERIAL BLOOD.
20 vol.% O_2
40 vol.% CO_2
TO TISSUES.
350 cc.
SUPPLEMENTAL AIR.
1500 cc.
RESIDUAL AIR 1500 cc.
CO_2 5.6 %. O_2 14.5%.
COMPLEMENTAL AIR.
2000 cc.

FIG. 195. INSPIRED AND EXPIRED AIR.

The *tidal air* is that which enters and leaves the body with each normal respiration. The *supplemental air* is that which can be forcibly expired after a normal tidal expiration; this leaves in the lungs the *residual air* which cannot be voluntarily expelled. The *complemental air* is that which can be forcibly drawn into the lungs over and above a normal tidal inspiration. The *vital capacity* is the greatest volume of air which can be expired following a forced inspiration; it includes the tidal, the supplemental, and the complemental volumes.

The *inspired* or atmospheric air is of constant composition. The composition of the *expired air* is not constant; it is determined by the varying factors of combustion in the tissues, the rate of blood flow, and the rate of ventilation in the lung. The *alveolar air* is the last portion of the supplemental air to be expired and represents the composition of the residual air, and its gases are in equilibrium with the arterial blood. The blood in its passage through the lung gives off carbon dioxide and absorbs oxygen.

Fig. 195 shows that after an ordinary expiration, 1500 ml. of additional air can be forcibly expired; then, if the greatest possible inhalation is made, almost 4 liters of fresh air can be drawn into the lungs. This greatly dilutes the carbon dioxide in the alveolar air and induces a rapid passage of the gas from the blood. If forced breathing is continued, a large amount of carbon dioxide, which is ordinarily stored in the blood, is in this manner "washed out" and may be erroneously regarded as carbon dioxide produced during a period of a metabolism test.

The increased supply of oxygen due to forced ventilation has no effect on metabolism tests; in fact, as has previously been stated, one can breathe pure oxygen without appreciably increasing its absorption. This fact is taken advantage of in the "closed circuit" types of respiration apparatus which require the rebreathing of air rich in oxygen.

Nitrogen plays a passive role in respiration; the amount expired is the same as the amount inspired. As there is a definite proportion between the oxygen and the nitrogen of the atmosphere, it is possible to calculate the amount of oxygen inspired on the basis of the nitrogen in the expired air. The formula is:

$$V = \frac{20.94 \times \text{per cent } N_2 \text{ in expired air}}{79.03}$$

when V = volume of oxygen in inspired air corresponding to 100 volumes of expired air.

It must be emphasized that in order to measure successfully the carbon dioxide produced during a metabolism test of short duration, *ventilation must be an involuntary act.*

Energy Requirement. The energy requirement of the animal body may be divided into two functional classifications, viz., that for basal metabolism and that for active work. Basal metabolism includes the energy expended in respiration, blood circulation, intestinal contractions, activities of various organs, maintenance of muscular tonus, thermal equilibrium, etc. The basal metabolic rate is influenced by the amount of active protoplasmic mass (hence by height, weight, surface area, age, sex, composition of the tissues, etc.) and is governed by endocrine organs, particularly the thyroid and pituitary glands. For a discussion of the clinical aspects of basal metabolism and its measurement, see p. 734.

The energy consumed in work, play, and indeed all forms of voluntary activity, imposes an additional requirement for fuel over the basal, which depends on the nature and extent of the muscular work. Whereas an average man expends about 100 Cal. per hour while sitting at rest, his metabolism may increase to as high as six times this value with extreme physical effort.

Mary Swartz Rose summarized the hourly expenditure of Calories of an average 70-kg. man under various conditions as follows: sleeping 65, awake lying still 77, sitting at rest 100, standing relaxed 105, walking slowly to moderately fast 200–300, running 5.3 miles per hour 570, swimming 500, walking down stairs 364, walking up stairs 1100, "light exercise" 170, "active exercise" 290, "very severe exercise" 600, dishwashing

144, carpentry or painting 240, sawing wood 480. From estimates such as these one may predict the calorific content of the diet necessary to meet individual requirements. An example for a 70-kg. man is given by Sherman as follows:

8 hrs. sleep at 65 Cal. = 520 Cal.
2 hrs. light exercise[3] at 170 Cal. = 340 Cal.
8 hrs. carpenter work at 240 Cal. = 1920 Cal.
6 hrs. sitting at rest at 100 Cal. = 600 Cal.
Total food requirement for the day, 3380 Cal.

The total energy requirement of different types of workers ranges from a minimum of 2000 to 2500 Cal. per day (white-collar workers) to a maximum of 4000 to 6000 (lumbermen, excavators, etc.), of which about 1400 to 1900 Cal. are consumed in basal metabolism and the balance in various forms of activity.

Food calories consumed in excess of an individual's energy requirements are deposited principally in the form of adipose tissue which is drawn on for energy when the dietary intake of calories falls below the expended level. A deficit of approximately 3500 calories is required to deplete the body of one pound of adipose tissue. When the fat depots are exhausted, as in acute inanition, muscle protein is used as a source of energy.

BASAL METABOLISM

Basal metabolism, or the basal metabolic rate, is an expression of the heat production of the body in complete mental and physical repose, and in the postabsorptive state. For a successful test the subject must be in a "comfortable" environment, if "too hot" or "too cold" the result will be abnormally high. The body temperature must be within the normal limits. DuBois estimates an increase in metabolism of 7.2 per cent for each degree Fahrenheit in fevers. The patient must be quiet and preferably experienced with the test. Mental activity alone has but a slightly elevating effect upon the heat production. Emotional excitation will raise the metabolism 20 per cent or more probably owing to the stimulation of the adrenal glands. The emotional factor constitutes the greatest source of error in basal metabolism determinations. It is not difficult to insure muscular repose and physical comfort, but it requires the utmost sympathy and tact to allay the apprehensions of the patient and insure physiological repose. A common practice is to test the patient repeatedly and to accept the lowest or the last result as the true basal rate; the fallacy of this is obvious when one considers that the number of such tests is usually determined by the endurance of the patient. DuBois conducts three or four tests and takes the average of the two lowest that show fair agreement. Even under favorable conditions it is possible, in unstable individuals, to get variations as high as 6 per cent in as many hours. These variations are physiological, not technical; the technique is more accurate than our ability to recognize or control emotional changes.

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[3] Going to and from work, for example.

Clinical Interpretation of Basal Metabolism. In 1893 the clinical use of calorimetry was presaged by Friedrich Müller, who observed that patients with Graves's disease lost weight and had a marked nitrogenous waste despite the fact that the diet was adequate to maintain a normal state of nutrition. This observation was soon verified by Magnus-Levy, who determined the now well-known action of the thyroid gland in regulating the rate of combustion in the body. It is true that other factors affect the rate of heat production and that certain well-recognized diseases are accompanied by metabolic changes, but *in the majority of cases variations in the basal metabolism can be interpreted as variations in the function of the thyroid gland.*

In an extensive study of basal metabolic rate determinations, Boothby and Sandiford showed that 92.1 per cent of normal individuals have a basal metabolic rate within ±10 per cent, and 99.3 per cent within ±15 per cent of the DuBois standards. They found that a smaller percentage of these same subjects had basal metabolic rates within the same limits when the Harris and Benedict standards were used. These standards are discussed in the following section.

In clinical cases the basal metabolism may vary from 40 per cent below to 130 per cent above the average normal. In this discussion a tabulation of the findings in various disorders is avoided because, standing alone, the values are misleading. If no other cause can be found for an abnormal heat production, the result may be cautiously attributed to a disordered activity of the thyroid gland. Glands other than the thyroid may affect the rate of cellular combustion, but at the present time we know of only two body substances which change the rate of heat production, namely, thyroxine and adrenaline. The latter has a rapid, but fleeting, stimulating effect; the effect of the former lasts for several weeks. When the activity of the adrenal glands is decreased, as in Addison's disease, the basal metabolism is found to be subnormal. Hyperactivity of these glands may account for the temporary increase in metabolism occasionally found in nervous patients, who also show corresponding variations in pulse rate and in blood pressure.

The basal metabolic rate does not establish a diagnosis of hyperthyroidism. A patient afflicted with hyperthyroidism may be tested during an intermission and the basal metabolism would be within the normal limits despite the persistence of such clinical signs as nervousness, palpitation, tremors, and exophthalmos. Conversely, the test may detect active hyperthyroidism in patients showing few of the classical symptoms of the disease. The test does not tell the surgeon when it is safe to operate upon the patient; it informs him when it is least dangerous. He must base his operative prognosis upon the clinical condition of the patient and not upon the basal metabolic rate.

The onset of hyperthyroidism is accompanied by a progressive increase in heat production which may reach over 100 per cent above the average normal and persist for weeks after the glandular activity has subsided. Therefore, repeated tests are necessary in order to follow the course of the disease; an increasing rate indicates an active state of the gland and naturally precludes operative intervention; a decreasing rate gives a more hopeful outlook.

The pulse rate is largely dependent upon the rate of metabolism. Clinically, a decreasing pulse rate indicates a subsidence of the hyperthyroidism. Few patients with pulse rates below 50 have a basal metabolic rate above the average normal and few patients with pulse rates below 85 have any considerable increase in their metabolism.

Heat comes from oxidation in the active protoplasmic tissue of the body. The rate of heat production is not determined by surface cooling, as one method of measurement would seem to imply (see footnote 17, p. 738); in reality, heat dissipation is regulated according to heat production. Any rapid change in the relative amount of protoplasmic tissue will cause changes in the basal metabolic rate when expressed in terms of weight or surface area. This partly explains the gradual drop in the basal metabolic rate during the glandular and muscular waste of starvation and in hospital patients during their early confinement to bed. A rapid accumulation of fluid in the body has a similar effect and it is quite common to find nephritics with marked edema having a basal metabolic rate 20 to 30 per cent below the average normal. This must not be interpreted as a sign of hypothyroidism.

Under the basal conditions we measure the heat resulting from activities of the vital organs and from the intracellular chemical changes associated with life, growth, and development. These facts must be borne in mind and due allowance made for any unusual activity before the results are attributed entirely to the thyroid gland. Dyspnea, hypertension, cardiac decompensation, and tremors involve increased muscular activity and so raise the heat production. Certain diseases are characterized by increased cellular activity and in these cases the heat production is increased. The most outstanding of this group is leukemia, in which disease the basal metabolic rate may be as high as in severe Graves's disease. Polycythemia, anemia, and Paget's disease of the bones are other conditions in which the increased heat production probably results from increased cellular activity. In this group possibly belongs the acute stage of acromegaly. In these conditions the basal metabolism is rarely over 25 per cent above the average normal. The possibility of self-administration of drugs such as iodine or thyroid extract must be considered in the interpretation of basal metabolic rate.

From birth to the age of one and a half years the basal metabolism increases at a remarkable rate. This is followed by a gradual decline until full growth and development are attained; constancy characterizes the rate in adult life, with a slight decline as old age advances.[4] Variations in the rate of growth and development in childhood cause abnormal results when judged by the age standard. In prematurely developed children the basal metabolic rate is low according to the age standard, but is probably normal for the stage of development if this could be accurately expressed. The difficulty in applying the test to children has resulted in a paucity of normal tests and great variation in the results obtained. The normal biological variations are much greater in children than in adults and may be greater than the pathological changes anticipated by the physician. Fortunately the test is rarely required before the age of puberty and the normal standards beyond that age are fairly well established.

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[4] Of interest in this connection are the observations of Benedict, Sherman, Campbell, and Zmachinsky (*J. Nutrition*, **14**, 179 (1937)) that middle-aged male rats not previously exercised were unable to adjust themselves to vigorous enforced exercise and declined rapidly, whereas female rats were benefited by similar treatment, showing a distinct tendency to lower basal metabolism. Basal metabolism in old age is the subject of a paper by Matson and Hitchcock: *Am. J. Physiol.*, **110**, 329 (1934).

The metabolism of women averages about 12 per cent below that of men, and owing to the menstrual cycle, is more variable. There is usually, though not invariably, a premenstrual rise and a postmenstrual fall in the basal metabolic rate, which should be considered in the interpretation of results.

There is some evidence of lower metabolic rates among certain oriental and tropical races.

Standards of Normal Basal Metabolism and Calculations.[5] The heat production of normal individuals under basal conditions largely depends upon the factors of age, height, and weight. The normal standards are based upon thousands of determinations in several centers of investigation. A better appreciation of basal conditions and the elimination of a high proportion of first tests has resulted in a tendency toward lower standards in recent years. At the present time there are three different systems of predicting the normal heat production.

1. *Aub and DuBois*[6] determine the heat production in relation to the surface area of the body; the surface area is estimated by the DuBois and DuBois[7] formula which is based on the height and weight:

$$A = Wt^{0.425} \times Ht^{0.725} \times 71.84$$

where A equals the area in sq. cm., Wt the weight in kg., and Ht the height in cm. In routine work the nomogram of Boothby and Sandiford[8] (Fig. 196) may be used for determining the surface area. Stoner[9] has computed tables of values for this formula in unit cm. and kg. intervals for the height range of 110 to 200 cm. and the weight range of 20 to 110 kg.

The original so-called Sage standards of Aub and DuBois have been modified on the basis of larger numbers of normal controls. Bailey's[10] table covers the ages from 4 to 65 in yearly intervals, while Boothby and Sandiford[11] give values by years from 5 to 19 and in 5-year intervals between 20 and 79 (see also table on p. 738). These standards fail to reflect the modern downward trend, but since they are based on tests on patients, they may be better suited for clinical purposes than lower standards based on trained subjects.

2. *The Harris-Benedict*[12] multiple prediction equations and tables[13] are based on

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[5] For prediction tables of normal heat production, reference should be made to books dealing in this subject (see the Bibliography at the end of this chapter).

[6] Aub and DuBois: *Arch. Internal Med.*, **19**, 823 (1917).

[7] DuBois and DuBois: *Arch. Internal Med.*, **17**, 865 (1916).

[8] Boothby and Sandiford: *Boston Med. Surg. J.*, **185**, 337 (1921).

[9] Stoner: *J. Lab. Clin. Med.*, **11**, 355 (1926).

[10] Bailey: *J. Lab. Clin. Med.*, **6**, 657 (1921).

[11] Boothby and Sandiford: *Am. J. Physiol.*, **90**, 290 (1929). See also Krogh's table in DuBois: *Basal Metabolism in Health and Disease*, 3d ed. Philadelphia, Lea & Febiger, 1936.

[12] Harris and Benedict: *Carnegie Inst. Wash., Pub. No.* 279, 1919.

[13] Tables which greatly simplify the use of these formulas may be found in Carpenter: *Carnegie Inst. Wash. Pub. No.* 303, 1921. See also DuBois's book, cited above.

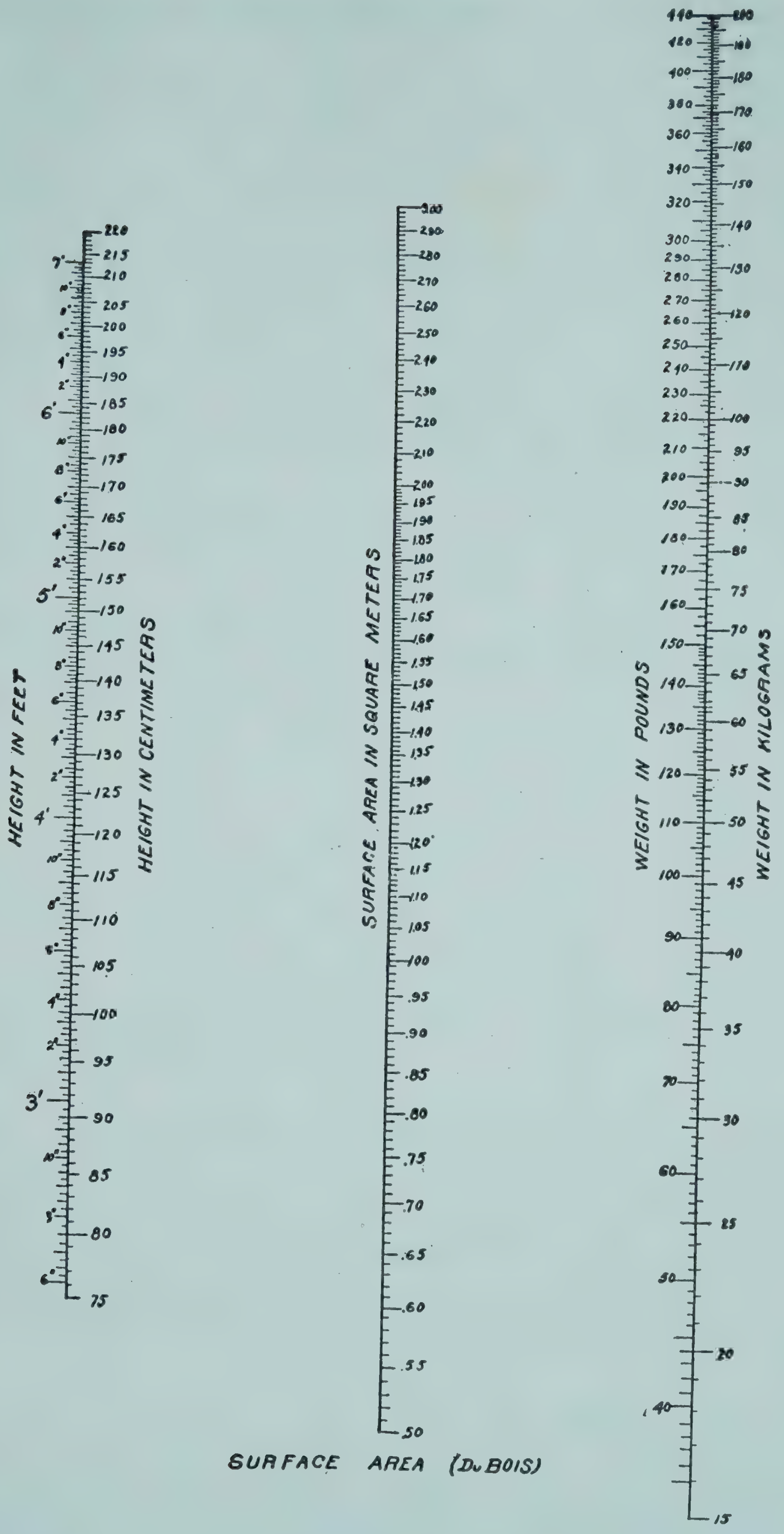

FIG. 196. SURFACE-AREA NOMOGRAM.

A line connecting the height with the weight intersects the middle line at the corresponding surface area.

Copyright 1920 by Boothby and Sandiford.

a statistical study of the available data for the basal metabolism of normal men and women. The equations are,

$$\text{For men,}\quad H = 66.4730 + 13.7516W + 5.0033S - 6.7550A$$
$$\text{For women,}\quad H = 655.0955 + 9.5634W + 1.8496S - 4.6756A$$

in which H = total heat production per 24 hours, W = weight in kilograms, S = stature in centimeters, and A = age in years. The Harris-Benedict standards average about 3 to 4 per cent lower than the Sage standards, and according to Benedict[14] they should be even lower, especially for women. He recommends lowering the standards for women by 5 per cent. Benedict calls attention to the distinction between "hospital normals" and physiological standards, the use of the former being justified for clinical purposes only.

STANDARD VALUES FOR MEAN ENERGY PRODUCTION IN RELATION TO AGE AND SEX*

Age at Last Birthday Year	*Calories per sq. m. per hr.* *Males*	*Females*
6	53.0	50.6
7	52.5	49.1
8	51.8	47.0
9	50.5	45.9
10	48.5	45.8
11	47.2	45.3
12	46.8	44.3
14	46.4	41.5
16	45.7	38.9
18	43.3	37.0
20	41.8	36.2
25	40.4	35.9
30	39.6	35.8
35	38.9	35.7
40	38.2	35.0
50	37.0	34.5
60	35.8	33.0

* After Boothby, Berkson, and Dunn: *Am. J. Physiol.*, **116**, 468 (1936).

These and other methods have been critically studied by Means and Woodwell,[15] and by Boothby and Sandiford,[16] and by Krogh. The consensus seems to be that the Harris-Benedict predictions are the most reliable for average normal subjects; that the DuBois standards give results in average normal cases about 4 per cent too high; that in all cases, including those of exceptional build and age, the DuBois standards show less deviation than any of the others.[17]

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[14] Benedict: *Am. J. Physiol.*, **85**, 607 (1928).

[15] Means and Woodwell: *Arch. Internal Med.*, **27**, 608 (1921).

[16] Boothby and Sandiford: *J. Biol. Chem.*, **54**, 767 (1922); *Physiol. Revs.*, **4**, 69 (1924).

[17] While the DuBois formula indicates that a definite mathematical relationship exists between the surface area and the quantity of energy produced, it has been disputed whether this is a true physiological relation. Benedict, the chief opponent of the surface-area law, suggests, however, that "From the practical standpoint it is perhaps not a matter of importance whether the rate of metabolism is directly proportional to the surface area, whether it is controlled by the heat loss from the body, whether the heat loss is independent of the heat supply, or whether the heat produced is determined by the active mass of protoplasmic tissue and the stimulus to the cells. But it is important to know whether there is a referable basis which can be used intelligently for comparing various individuals." *Lectures on Nutrition*, p. 51, Philadelphia, W. B. Saunders Co., 1925.

The method of predicting the normal heat production, and the normal standards, for infants and children are the subjects of several papers.[18]

THE RESPIRATORY QUOTIENT (R.Q.). This is the ratio of the volume of carbon dioxide produced to the volume of oxygen consumed during the same time interval.

$$\text{R.Q.} = \frac{\text{CO}_2 \text{ produced}}{\text{O}_2 \text{ consumed}} = \frac{\text{volume CO}_2 \text{ in expired air} - \text{volume CO}_2 \text{ in inspired air}}{\text{volume O}_2 \text{ in inspired air} - \text{volume O}_2 \text{ in expired air}}$$

The measurement of the volume of inspired air is attended with technical difficulties. However, the actual volumes of gas disappear from the working formula as demonstrated below. If we let

V_e and V_i = volume of expired and inspired air
C_e and C_i = per cent CO_2 in expired and inspired air
O_e and O_i = per cent O_2 in expired and inspired air
N_e and N_i = per cent N_2 in expired and inspired air

we may rewrite the above equation as follows:

$$\text{R.Q.} = \frac{C_eV_e - C_iV_i}{O_iV_i - O_eV_e} \tag{1}$$

Since nitrogen is neither absorbed nor evolved, its volume in inspired and expired air remains unchanged. Therefore its percentage in inspired and expired air is in inverse ratio to their respective volumes. That is

$$N_i : N_e = V_e : V_i$$

or

$$V_i = \frac{N_eV_e}{N_i}$$

Substituting this value in *(1)* and simplifying, we get

$$\text{R.Q.} = \frac{C_eN_i - C_iN_e}{O_iN_e - O_eN_i} \tag{2}$$

Substituting the values given for the composition of atmospheric air (p. 730) for O_i, C_i and N_i, we obtain

$$\text{R.Q.} = \frac{79.03C_e - 0.03N_e}{20.94N_e - 79.03O_e} \tag{3}$$

Simplifying,

$$\text{R.Q.} = \frac{C_e - 0.00038N_e}{0.265N_e - O_e}$$

Since $0.00038N_e$ never varies significantly from 0.03, we have finally

$$\text{R.Q.} = \frac{C_e - 0.03}{0.265N_e - O_e} \tag{4}$$

From this equation it is possible to calculate R.Q. simply from the CO_2 and O_2 percentages in expired air, since N_e is obtained by difference.

BASAL METABOLISM OR BASAL METABOLIC RATE (B.M.R.). This may be defined as the percentile variation of the observed from the normal or predicted heat production for an individual of given height, weight, age, and sex in a postabsorptive state and in complete mental and physical repose. The heat production for a given period is obtained by multiplying the volume, at normal temperature and pressure, of oxygen consumed during that period by the calorific value for oxygen corresponding to the observed (or assumed) R.Q. Basal heat production may be expressed on an hourly or daily basis. The general formula for basal metabolic rate is therefore

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[18] Talbot: *Physiol. Revs.*, **5**, 477 (1925); Stark: *Am. J. Physiol.*, **111**, 630 (1935); Talbot, Wilson, and Worcester: *Am. J. Diseases Child.*, **53**, 273 (1937); Lewis, Kinsman, and Iliff: *Am. J. Diseases Child.*, **53**, 348 (1937).

$$\text{B.M.R.} = \underset{\substack{\textit{Corrected volume}\\ \textit{of } O_2 \textit{ consumed}\\ \textit{in } m \textit{ minutes}}}{\frac{273pv}{760t}} \times \underset{\substack{\textit{Factor for con-}\\ \textit{version to}\\ \textit{hourly (or daily)}\\ \textit{basis}}}{\frac{60(\text{or } 1440)}{m}} \times \underset{\substack{\textit{Cal.}\\ \textit{per}\\ \textit{liter}\\ O_2}}{C} \times \underset{\substack{\textit{Ratio}\\ \textit{to}\\ \textit{normal}\\ \textit{heat}\\ \textit{produc-}\\ \textit{tion}}}{\frac{1}{N}} \times \underset{\substack{\textit{Conver-}\\ \textit{sion to}\\ \textit{per}\\ \textit{cent}\\ \textit{basis}}}{100} - \underset{\substack{\textit{Assuming}\\ \textit{normal}\\ \textit{heat pro-}\\ \textit{duction}\\ \textit{to be}\\ \textit{100 per}\\ \textit{cent}}}{100} \qquad (5)$$

In this equation, p is the barometric pressure in mm. of mercury corrected for tension of aqueous vapor; v the observed volume, in liters, of oxygen consumed during the basal test period; m the duration of the basal test period in minutes; t the absolute temperature (° C + 273); C the calorific value per liter of oxygen[19] corresponding to the observed (or assumed) respiratory quotient (see the table on p. 727; in the oxygen consumption methods the R.Q. is assumed to be 0.82, which corresponds to a calorific value per liter of oxygen of 4.825); and N, the normal or predicted basal heat production obtained from one of the various tables of standards.

In the usual form of oxygen consumption apparatus equipped with volumetric scales and CO_2 absorbents, v in equation (5) is obtained by subtracting the reading at the end from that at the beginning of the test period.

When the gasometric method is employed,

v = volume O_2 in inspired air − volume O_2 in expired air, or

$$v = 0.01O_iV_i - 0.01O_eV_e \qquad (6)$$

By substituting the value for V_i as above, and simplifying,

$$v = 0.01V_e\left(O_i\frac{N_e}{N_i} - O_e\right)$$

in which V_e is the gasometer reading, R, multiplied by the gasometer factor, f, hence the volume of expired air in liters. By further substitution of the values for O_i and N_i given above, and simplifying,

$$v = 0.01Rf(0.265N_e - O_e)$$

in which the variables are R, N_e, and O_e. This expression for v may be incorporated in equation (5).

The *Aub and DuBois tables* give the normal heat production in Cal. per sq. m. of body surface per hr. (A), which when multiplied by the surface area in sq. m. (S) gives the predicted *hourly* heat production. Making the proper substitutions in the above equation, and collecting constants, we obtain the following simplified formula:[20]

$$\text{B.M.R.} = \frac{2155pvV}{tmSA} - 100$$

In the *Harris and Benedict tables* for normal heat production, the values are expressed on a 24-hour basis. The coefficient in the equation becomes therefore 24 × 2155 = 51720, and the equation is

---

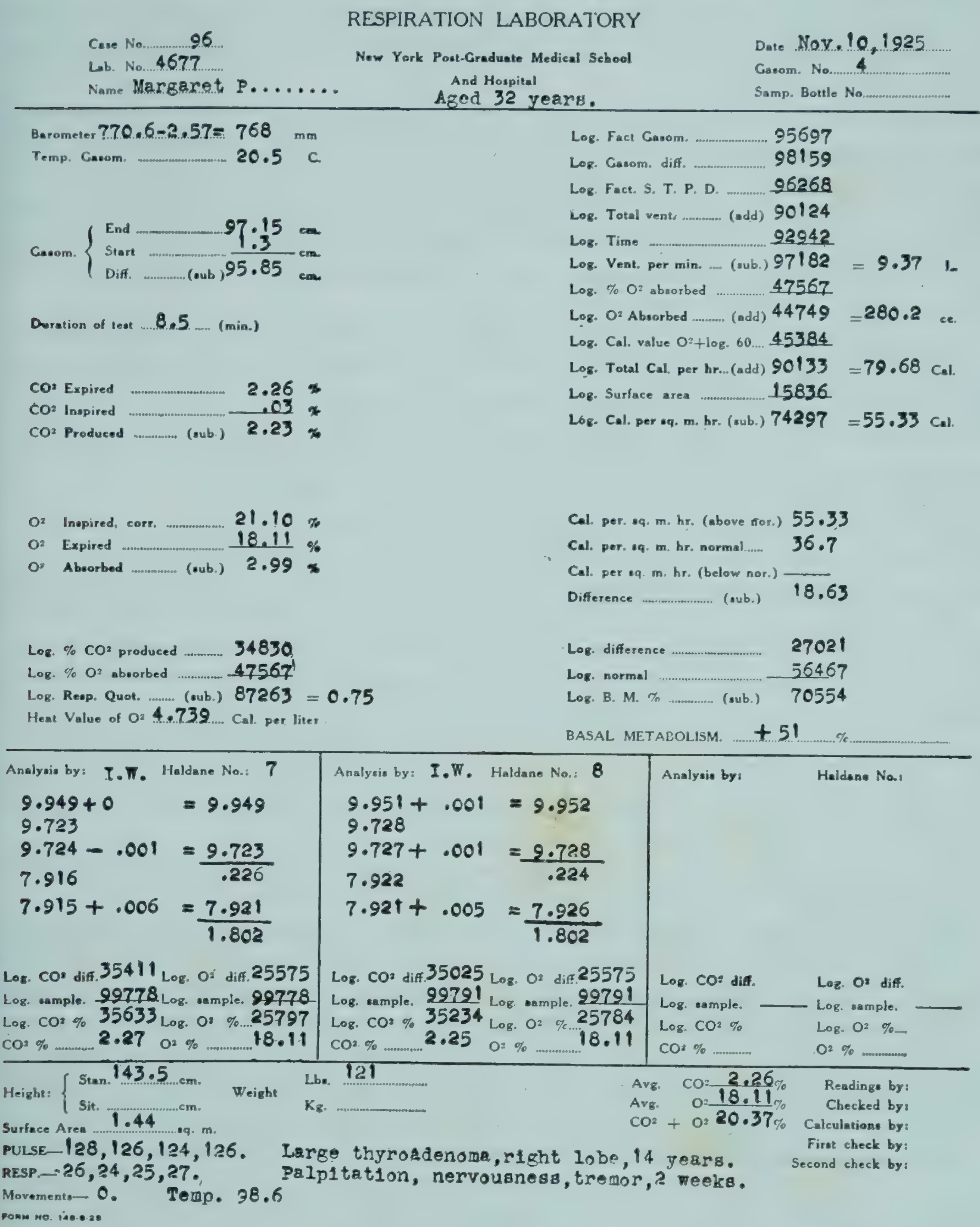
[19] According to Magnus-Levy, the metabolism of protein furnishes on the average 15 per cent of the total calories. This may be taken into account by using figures for the calorific value of 1 liter of oxygen 1 per cent lower than those given in the table on p. 727.

[20] Stoner (*Boston Med. Surg. J.*, **189**, 195 (1923); *J. Lab. Clin. Med.*, **12**, 884 (1927); **13**, 164 (1927)) has described these and other simplifications in the calculations for respiratory exchange, as well as data cards which are very useful in routine practice.

$$\text{B.M.R.} = \frac{51720pvC}{tmH} - 100$$

in which H represents the predicted 24-hour heat production.

In the oxygen consumption methods R.Q. is assumed to be 0.82. Hence 4.825 is substituted for C, in the above equations (see the table on p. 727) and the coefficients become 10,400 and 250,000 respectively. Further simplification is attained in routine practice by standardizing the basal test period and merging the value for m into the coefficient.



RESPIRATION LABORATORY

Case No. 96
Lab. No. 4677
Name Margaret P........

New York Post-Graduate Medical School
And Hospital
Aged 32 years.

Date Nov. 10, 1925
Gasom. No. 4
Samp. Bottle No.

Barometer 770.6-2.57= 768 mm
Temp. Gasom. 20.5 C.

Gasom. { End 97.15 cm. / Start 1.3 cm. / Diff. (sub) 95.85 cm.

Duration of test 8.5 (min.)

CO^2 Expired 2.26 %
CO^2 Inspired .03 %
CO^2 Produced (sub.) 2.23 %

O^2 Inspired, corr. 21.10 %
O^2 Expired 18.11 %
O^2 Absorbed (sub.) 2.99 %

Log. % CO^2 produced 34830
Log. % O^2 absorbed 47567
Log. Resp. Quot. (sub.) 87263 = 0.75
Heat Value of O^2 4.739 Cal. per liter

Log. Fact Gasom. 95697
Log. Gasom. diff. 98159
Log. Fact. S. T. P. D. 96268
Log. Total vent. (add) 90124
Log. Time 92942
Log. Vent. per min. (sub.) 97182 = 9.37 l.
Log. % O^2 absorbed 47567
Log. O^2 Absorbed (add) 44749 = 280.2 cc.
Log. Cal. value O^2+log. 60 45384
Log. Total Cal. per hr. (add) 90133 = 79.68 Cal.
Log. Surface area 15836
Log. Cal. per sq. m. hr. (sub.) 74297 = 55.33 Cal.

Cal. per. sq. m. hr. (above nor.) 55.33
Cal. per. sq. m. hr. normal 36.7
Cal. per sq. m. hr. (below nor.) ——
Difference (sub.) 18.63

Log. difference 27021
Log. normal 56467
Log. B. M. % (sub.) 70554

BASAL METABOLISM. +51 %

Analysis by: I.W. Haldane No.: 7	Analysis by: I.W. Haldane No.: 8	Analysis by: Haldane No.:
9.949 + 0 = 9.949	9.951 + .001 = 9.952	
9.723	9.728	
9.724 — .001 = 9.723	9.727 + .001 = 9.728	
.226	.224	
7.916	7.922	
7.915 + .006 = 7.921	7.921 + .005 = 7.926	
1.802	1.802	
Log. CO^2 diff. 35411 Log. O^2 diff. 25575	Log. CO^2 diff. 35025 Log. O^2 diff. 25575	Log. CO^2 diff. Log. O^2 diff.
Log. sample. 99778 Log. sample. 99778	Log. sample. 99791 Log. sample. 99791	Log. sample. —— Log. sample. ——
Log. CO^2 % 35633 Log. O^2 % 25797	Log. CO^2 % 35234 Log. O^2 % 25784	Log. CO^2 % Log. O^2 %
CO^2 % 2.27 O^2 % 18.11	CO^2 % 2.25 O^2 % 18.11	CO^2 % O^2 %

Height: { Stan. 143.5 cm. / Sit. cm.
Weight Lbs. 121 / Kg.
Surface Area 1.44 sq. m.

Avg. CO^2 2.26 %
Avg. O^2 18.11 %
CO^2 + O^2 20.37 %

Readings by:
Checked by:
Calculations by:
First check by:
Second check by:

PULSE—128,126,124,126.
RESP.—26,24,25,27.
Movements— 0. Temp. 98.6

Large thyroadenoma, right lobe, 14 years.
Palpitation, nervousness, tremor, 2 weeks.

FORM NO. 148-8-25

FIG. 197. FORM SHEET FOR STUDY OF RESPIRATORY METABOLISM.

The calculations may be performed either on calculating machines or logarithmically, in which case five-place tables are used and the characteristics omitted, since the decimal place may be pointed off in the final result by inspection. A form sheet for logarithmic computation described by Boothby and Sandiford is shown in Fig. 197.

The calculations involved in the interpretation of *kymographic records* of respiratory exchange are described in the legend to Fig. 200.

HUMAN AND ANIMAL CALORIMETRY. The rate of combustion may be determined by directly measuring the heat given off by the body. Every animal has an optimum temperature and this temperature is maintained by a nice balance between the heat produced by the life processes and the heat lost by the organism.[21] Gephart and DuBois estimate that in man, 24 per cent of the total heat loss results from the evaporation of water from the lungs and skin. A small amount of heat is lost in warming the ingested food, the urine, the feces, and at times the body itself; the remainder is lost by conduction and by radiation.

Lavoisier and Laplace measured the heat loss by placing a guinea pig in an ice chamber for ten hours and observing the amount of ice melted. Over a period of many years, the elaboration of this simple form of calorimeter has culminated in the construction of the Atwater-Rosa-Benedict Respiration Calorimeter.[22] With this apparatus, the heat of the body is determined indirectly from the respiratory exchange, and directly by a careful computation based on observations of all the known means of heat loss.

The principle of this apparatus was applied by Armsby[23] in the elaboration of a calorimeter for use with farm animals. Benedict[24] and associates also devised a respiration chamber for use with domestic animals. This apparatus was later modified for use in human calorimetry.[25] Respiration calorimeters have provided the means of acquiring most of the present-day knowledge of energy metabolism. They have been extremely valuable in proving the accuracy of indirect calorimetry.

Because of the expense of installation and the technical difficulties of operation of respiration calorimeters, heat production is usually measured indirectly, i.e., from the gaseous exchange. Two types of methods are in use: (1) Open-circuit methods, in which atmospheric air is breathed and expired air collected and analyzed; and (2) closed-circuit methods, in which oxygen-enriched air is breathed and the consumed oxygen measured. The former procedure provides a more complete picture of respiratory exchange since it permits determination of the respiratory quotient. On the other hand, the closed circuit methods are much simpler technically and where only total metabolism must be determined are equally satisfactory. In the closed circuit method an average respiratory quotient of 0.82, corresponding to a calorific value for O_2 of 4.825 Cal. per liter, is assumed.

The various types of respiration apparatus on the market are simplified modifications of Dr. F. G. Benedict's[26] clinical respiration chamber.

Basal metabolism is usually determined in 10- to 15-minute periods using either one of the Benedict closed-circuit methods as developed in the Carnegie Nutrition Laboratory, or a modified Tissot or open-circuit method. Obviously, the determination of urinary nitrogen in such tests is impracticable. It has been the custom to apply the calorific values in the Zuntz and Schumburg table (see p. 727) directly to the respiratory exchange as measured, without computing separately the protein metabolized. DuBois points out that this procedure gives results about 1 per cent too high.

The example described below serves to illustrate the basic principles employed in

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[21] For a review, see DuBois: *Harvey Lect.*, **34**, 88 (1939).

[22] Atwater and Rosa: *Report of Storr's Agric. Exp. Sta.*, 1897, p. 212; Atwater and Benedict: *Carnegie Inst. Wash. Pub.* No. 42, 1905.

[23] Armsby and Fries: Bull. 51, U. S. Dept. of Agriculture (Bureau of Animal Industry), 903.

[24] Benedict, Coropatchinsky, and Ritzman: *Abderhalden's Handbuch der biologischen Arbeitsmethoden*, 4 (part 13), 619, 1934.

[25] Newburgh, Johnston, Wiley, Sheldon and Murrill: *J. Nutrition*, **13**, 193 (1937).

[26] Later developments in closed-circuit apparatus and comparison with open-circuit methods are discussed by Benedict: *Boston Med. Surg. J.*, **193**, 807 (1925).

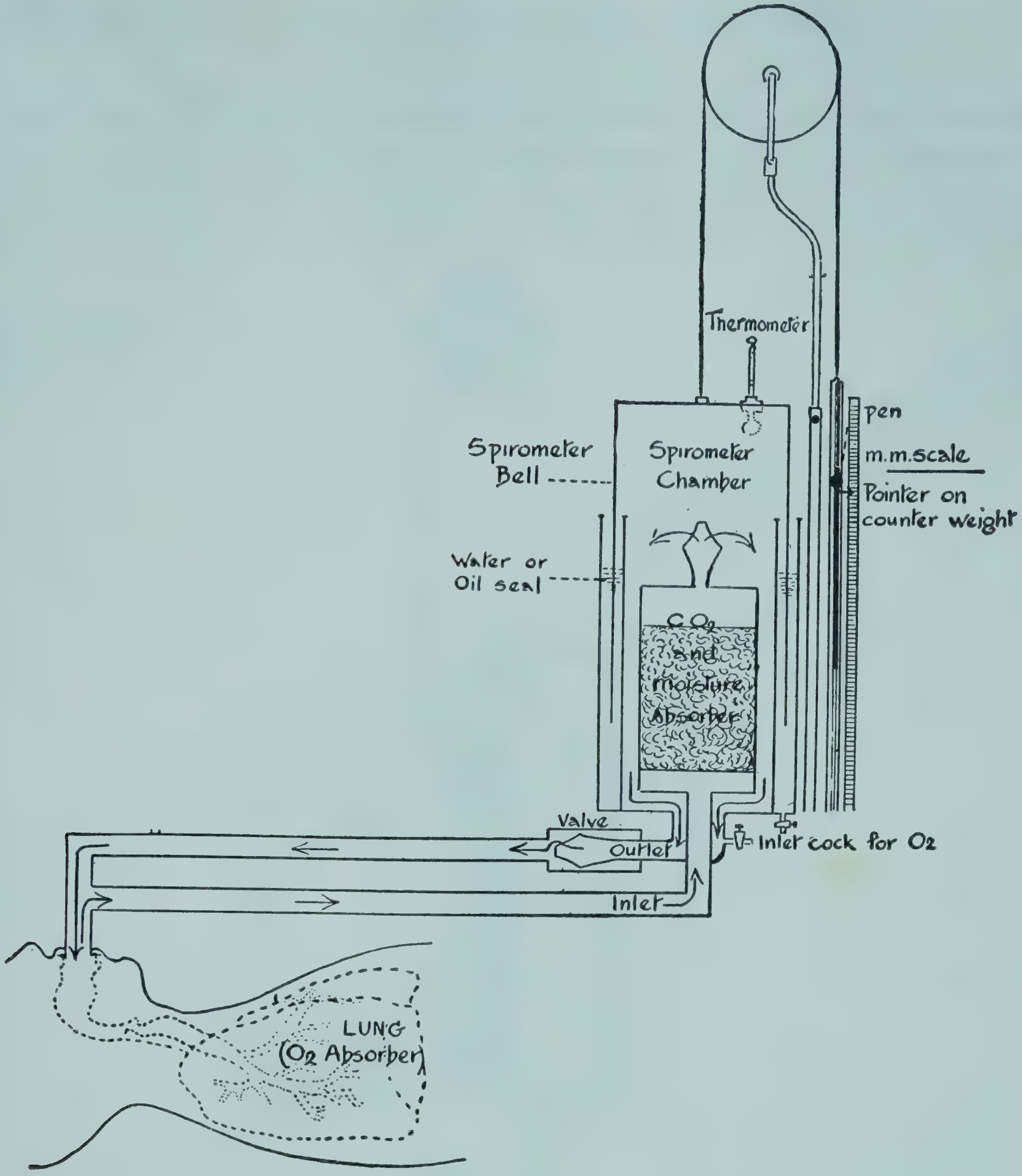
Thermometer
pen
m.m.scale
Pointer on counter weight
Spirometer Bell
Spirometer Chamber
Water or Oil seal
CO2 and moisture Absorber
Valve
Outlet
Inlet cock for O2
Inlet
LUNG (O2 Absorber)

FIG. 198. DIAGRAM OF THE BENEDICT-ROTH RESPIRATION APPARATUS.

The patient breathes through a mouthpiece. The nose is clamped. Two tubes connect the mouthpiece with the spirometer. An inspiratory Sadd valve directs the oxygen from the spirometer chamber to the patient; an expiratory valve insures its continuous circulation. The expirations pass through soda lime which removes carbon dioxide. The calibrated spirometer bell is balanced by a counterweight which carries a pointer. The pointer shows the movements of the bell on a millimeter scale. A test is started with the chamber full of oxygen; as oxygen is absorbed by the patient the volume in the chamber decreases. The loss of volume represents oxygen absorbed during the period of the test. Correction is made for any change in chamber temperature during the test.

Slightly modified from Roth: *Boston Med. Surg. J.*, **186**, 491 (1922).

the oxygen-consumption type of measurement. Specific directions are furnished with various commercial instruments adapted for office or hospital use.

The *Benedict-Roth respiration apparatus* (Figs. 198, 199) is extensively used in clinics for determining the basal metabolic rate of patients. Roth[27] has dispensed with the electric blower of the Benedict machines, and has inserted two Sadd valves to insure circulation of the oxygen. The spirometer bell is designed to have a volume of 20.73 ml. for every millimeter of height. This greatly simplifies the calculations. The fall of the spirometer bell during a test of 6 minutes represents the volume of oxygen absorbed by the patient. As with other Benedict apparatus, the respiratory quotient is assumed to be 0.82; this gives a heat value for 1 liter of oxygen of 4.825 Cal. "With a bell of this size, *each mm. of the fall of the bell in a six-minute period represents exactly 1 Cal. per hour.* (1 mm. in 6 min. = 20.73 ml. = 0.2073 liter per hour. 0.2073 × 4.825 = 1 Cal. per hour.)"

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[27] Roth: *Boston Med. Surg. J.*, **186**, 457, 491 (1922). This and other types of basal-metabolism apparatus for clinical use are made by Warren E. Collins, Inc., 553H Huntington Ave., Boston, Mass.: Sanborn Co., 39 Osborn St., Cambridge, Mass.; and Jones Metabolic Equipment Co., 1870 Ogden Ave., Chicago, Ill.

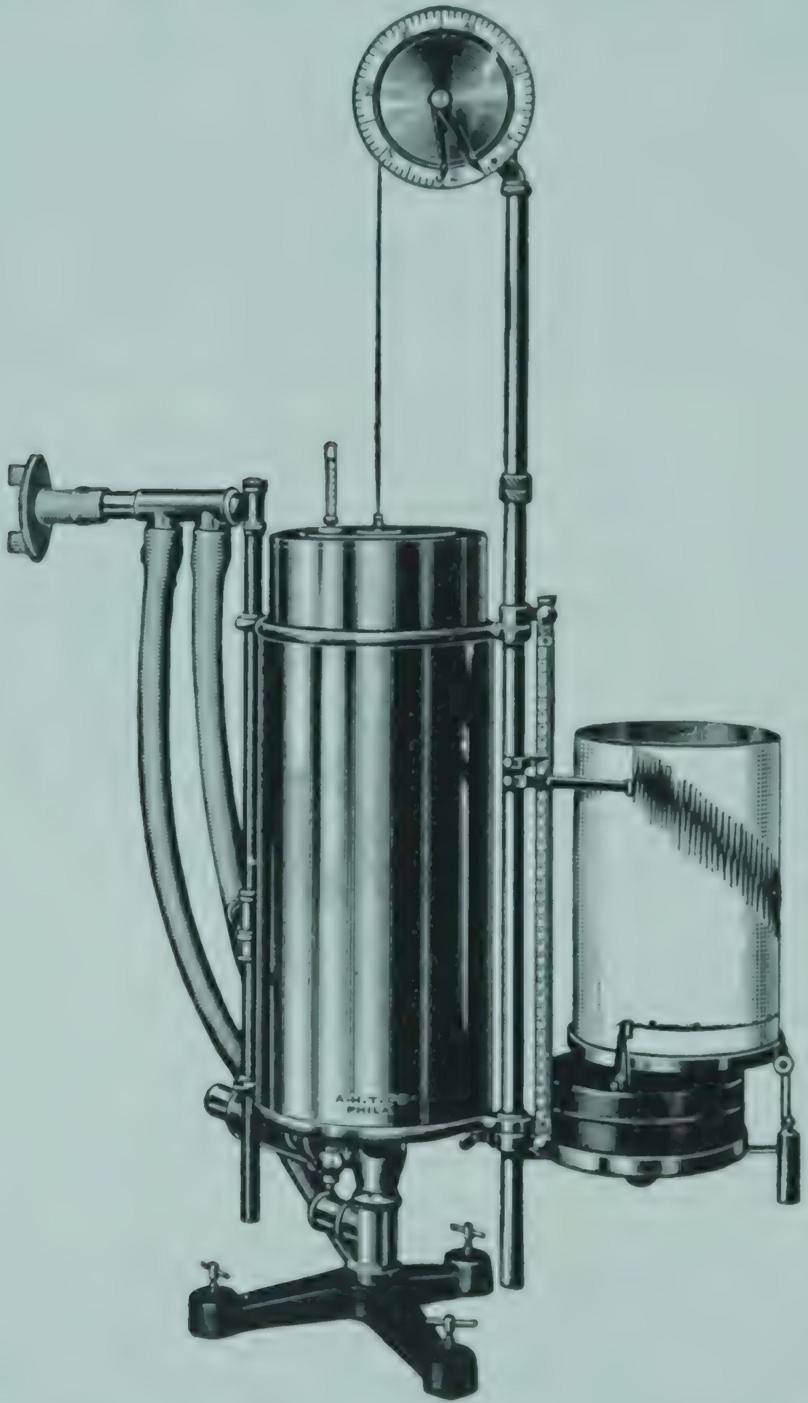

FIG. 199. BENEDICT-ROTH RESPIRATION APPARATUS WITH KYMOGRAPH.

The form of this apparatus illustrated in Fig. 199 is provided with a kymograph which is self-time-marking in minute periods. A pen attached to the counterweight traces the respiratory excursions and shows the lowering of the spirometer bell due to the absorption of oxygen. Fig. 200 illustrates the method of drawing the "oxygen consumption line," and of measuring the "rise" of this line for any 6-minute period which may be selected on the tracing. If the moisture content of the circulating air is high, as is the case when Wilson soda lime is used as an absorber, a correction must be made for water vapor. Roth[28] gives the factors for reducing the volume of absorbed oxygen to 0° C., 760 mm., dry (when the vapor tension is 80 per cent of saturation).

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[28] A table for reducing volumes of 80 per cent saturated air to 0° C. and 760 mm., dry, is given by Roth: *Boston Med. Surg. J.*, **186**, 457 (1922).

Procedure. **The patient is tested before breakfast and after a 12- to 15-hour fast. A record is made of the sex, age, height, and weight. A rest in a semi-reclining position for half an hour must precede the test. During the rest period, the pulse rate, the respiration rate and the temperature are observed, and the patient is briefly instructed in order to secure his confidence and cooperation. The apparatus is tested for leaks as follows: a stopper is placed in the opening of the mouthpiece, a 200-g. weight is placed on the bell. The pointer should remain stationary for a minute or more. The weight and stopper are removed and the spirometer is filled with oxygen. The mouthpiece and later the nose clip are applied to the patient, care being taken to avoid leaks. The kymograph[29] is started. The temperature of the spirometer, and the barometric pressure are recorded. The test is continued until a**

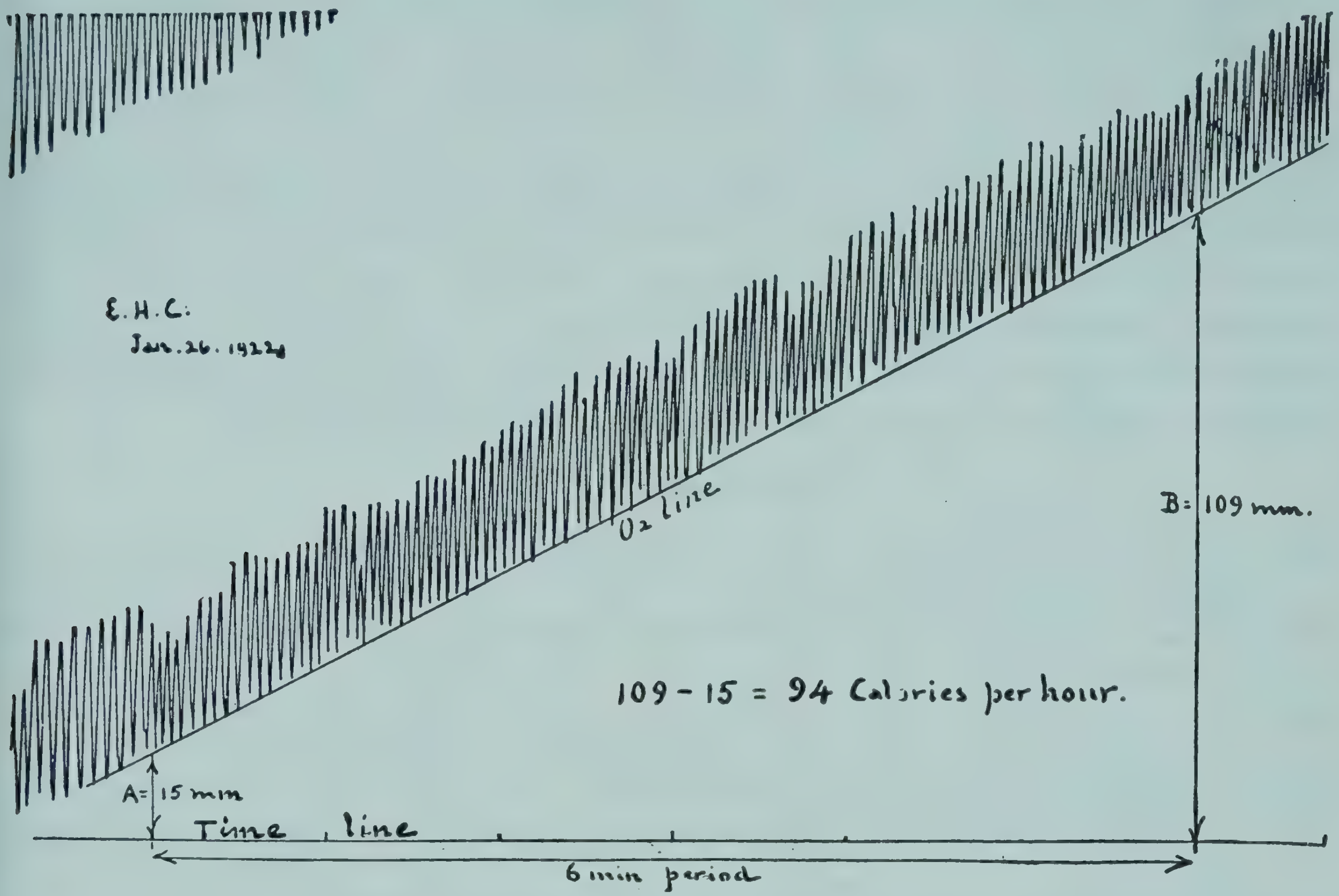

FIG. 200. GRAPHIC RECORD OF A METABOLISM TEST.

E. H. C., male, aged 38. Height, 176 cm. Weight, 84 kg. Surface area, 2.0 sq. m. Barometric pressure, 745 mm. Average temperature, 24° C. (no increase during test). Rise of O_2 line in 6 minutes, 94 mm. = 94 Cal. per hour. Correction for temperature, pressure, and water vapor 94 × 0.879 = 82.63 Cal. per hour. Divide by surface area 82.63 ÷ 2 = 41.3 Cal. per square meter of surface per hour, which is 5 per cent above the average normal (39.5) for this individual (Roth).

For an example of a simplified data card for taking records of instruments not equipped with kymographs and for making the calculations, see Stoner: *Boston Med. Surg. J.*, **189**, 195 (1923).

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[29] In the case of spirometers not equipped with kymographs, readings on the scale are made at the beginning and end of the periods. Stoner (*Boston Med. Surg. J.*, **189**, 193 (1923)) divides a 10-minute period into five 2-minute periods, taking the average of 10 spirometer readings at the beginning of each period. In this way he is able to select a 4-minute period showing the least oxygen consumption, and also to avoid the error due to wide fluctuations in individual respirations.

satisfactory uninterrupted section of exactly 6 minutes can be selected for subsequent measurement. Before stopping the test, a weight of 50 g. is placed on the bell and operation is continued for a few minutes; if a leak occurs it is shown by a sharp rise in the oxygen consumption line. The temperature of the spirometer is again recorded. Fig. 200 shows a graphic record of a test, and the legend gives the necessary calculations for determining the basal metabolic rate.

Wesson[30] has described an apparatus and procedure for determining the respiratory quotient in small animals. A multiple-chamber respiration apparatus for small animals, based on the oxygen-consumption method, has been described by Benedict.[31]

For research purposes the expired air may be collected in a gasometer or in an impervious rubber or plastic bag and subjected to analytical determination of CO_2 and O_2 content. Methods based on the use of the Haldane-Henderson gas-analysis apparatus are employed. These procedures offer greater flexibility and more information but require more expensive equipment and a high degree of manipulative skill.

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Sherman: *Chemistry of Food and Nutrition*, 8th ed. New York, The Macmillan Co., 1952.

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[30] Wesson: *J. Biol. Chem.*, **73,** 499 (1927).

[31] Benedict: *J. Nutrition*, 3, 161 (1930). This apparatus can be secured from Warren E. Collins, Inc., 553H Huntington Ave., Boston, Mass.

26

Hormones

The endocrine organs, or glands of internal secretion (see Fig. 201), are tissues whose function it is to secrete certain specific chemical substances known as *hormones* into the blood stream, which distributes them to all parts of the body. Certain tissues respond to their presence in a characteristic way, increasing, decreasing, or modifying their processes of growth or of metabolic or physiological activity. The hormones are therefore chemical messengers serving to integrate the various activities of the body; they may themselves undergo metabolic transformations, such as partial or complete oxidation, or reduction; and recognizable products of these transformations, or the hormones themselves, may be excreted in the urine and in the bile.

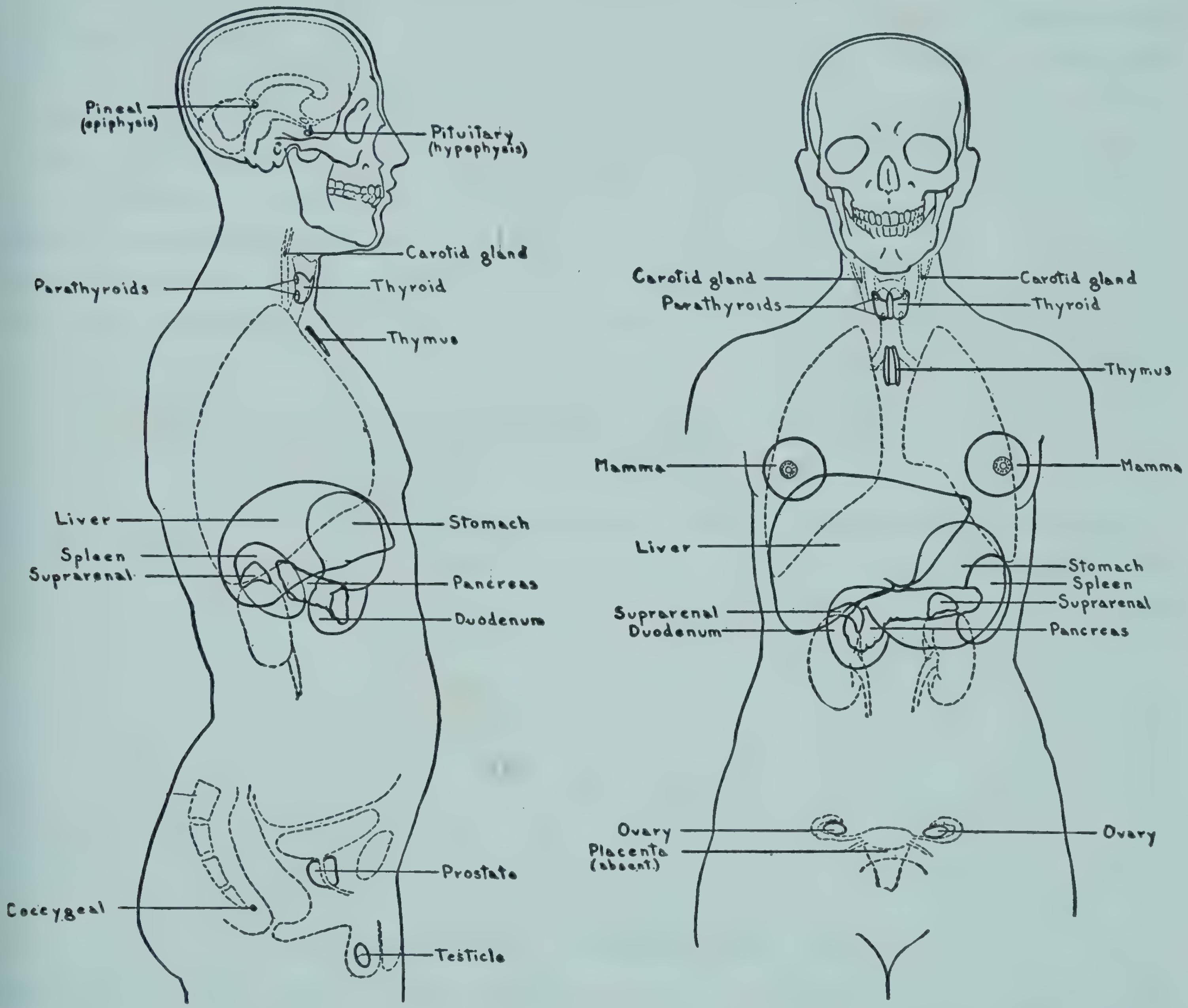

Pineal (epiphysis)
Pituitary (hypophysis)
Carotid gland
Parathyroids
Thyroid
Thymus
Liver
Stomach
Spleen
Suprarenal
Pancreas
Duodenum
Prostate
Coccygeal
Testicle
Carotid gland
Carotid gland
Parathyroids
Thyroid
Thymus
Mamma
Mamma
Liver
Stomach
Spleen
Suprarenal
Suprarenal
Duodenum
Pancreas
Ovary
Ovary
Placenta (absent.)

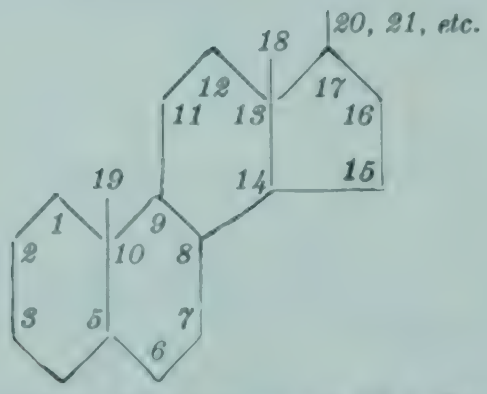
FIG. 201. SCHEMATIC CHART OF ENDOCRINE SYSTEM.
From Barker, *et al.: Endocrinology and Metabolism,* New York, Appleton, 1924.

The secretory activity of the endocrine organs varies from time to time. As the chart on p. 750 shows, the morphological structure and the endocrine activity of the gonads, adrenal cortex, and thyroid are largely controlled by hormones produced by the anterior lobe of the pituitary (anterior hypophysis) which also produces hormones acting directly on the structure and function of nonendocrine tissues. The anterior pituitary in turn is partly controlled (directly or indirectly) by the concentration of hormones produced by the gonads, thyroid, etc., and also by the nervous system directly. Similarly, the posterior pituitary and the adrenal medulla are subject to direct nervous regulation. The control of the endocrine activity of the pancreas, parathyroids, and other possible sources of hormones is not fully understood. It will be noted in the chart that a single organ, or a single metabolic process, may be affected by more than one hormone, and often also by factors not endocrine in nature.

The hormones do not vary significantly in chemical structure from species to species, hence extracts from the endocrine organs of animals may often be used in treating disorders due to insufficient activity of a human gland; synthetic or artificial products, allied to or identical with the natural hormones, may be less costly. Disorders due to excessive activity—for example, when a tumor develops from endocrine cells—are often treated by surgical removal of the abnormal tissue; or, more rarely, by selective inhibitory drugs or by irradiation with x-rays. Chemically, the known hormones may be divided into two main groups. The first may be referred to as nitrogenous, and range in size and complexity from simple bases such as adrenaline to proteins of high molecular weight and undetermined structure. The second group, more homogeneous, may be called the steroid hormones

THE STEROID HORMONES OF THE GONADS AND ADRENAL CORTEX

General Chemistry of the Steroids and Steroid Hormones. The basic saturated carbon ring skeleton of the steroids is illustrated in Formula I.



Carbon Ring Skeleton of the Steroids (I)

Depending on the chemical nature of the substituent (R) at C_{17}, the steroids may be divided into five classes:

(*a*) The *sterols*, like cholesterol and ergosterol (see Chapters 11 and 35), where R consists of an eight- or nine-carbon-atom aliphatic side chain.

(*b*) The *bile acids* (p. 410), where R consists of a five-carbon side chain terminating in a carboxylic acid group.

(*c*) The *cardiac aglycones*, breakdown products of the heart-stimulating glycosides of *Strophanthus*, *Digitalis*, etc., which are characterized by a lactone ring substituted at C_{17}.

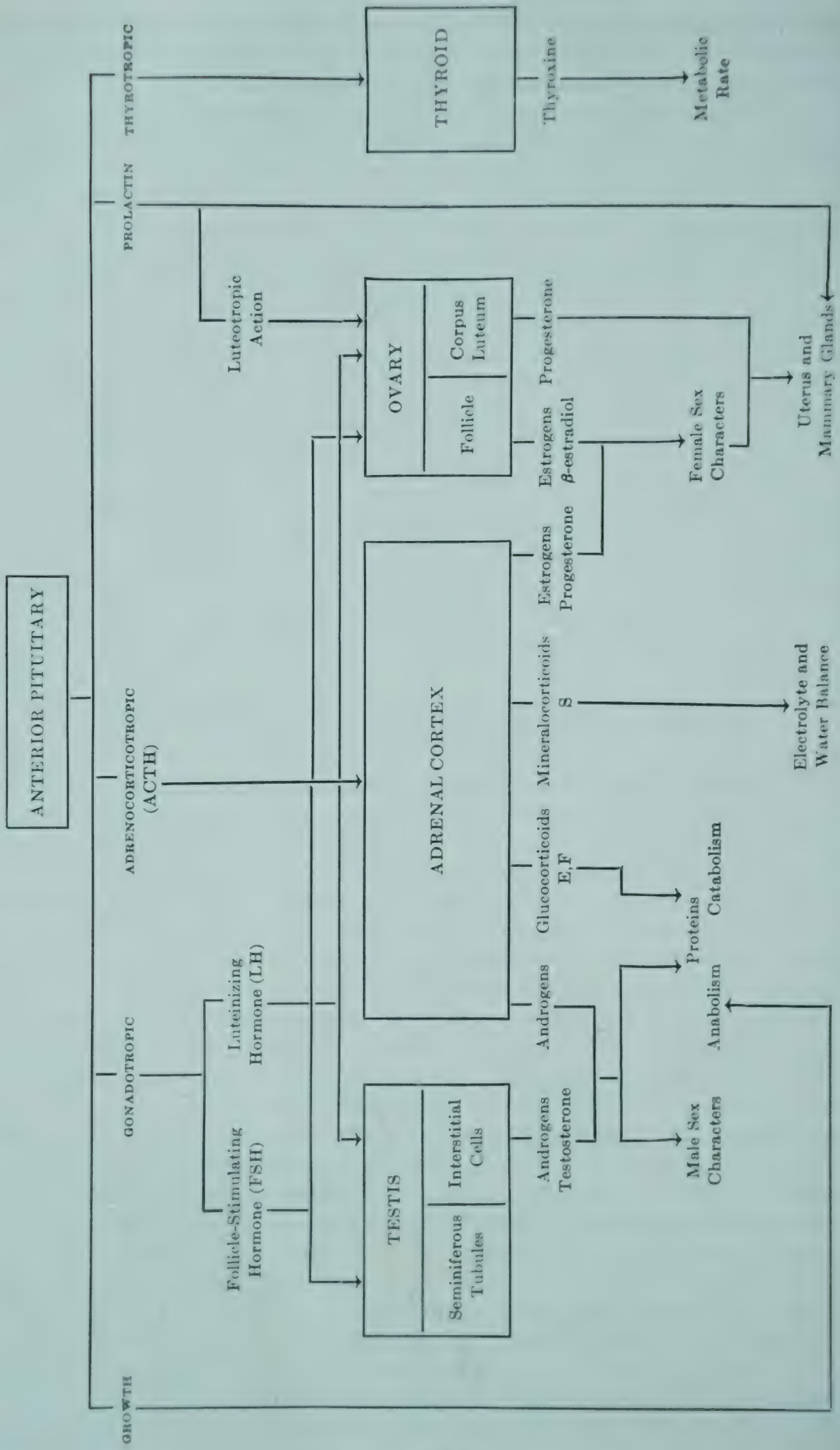
(*d*) The *sapogenins*, derived from plant saponins, where R is comprised of an ethereal ring system.

(*e*) The *steroid hormones*, where C_{17} bears a ketonic or hydroxyl group (the androgens and estrogens), or carries a substituted two-carbon side chain (progesterone and the adrenal cortical steroids).

The steroid hormones all carry phenolic or ketonic oxygen on C_3. The summary on pp. 751–754 formulates the principal steroid hormones, and illustrates, by means of arrows, the metabolic pathways followed in the body in the course of their inactivation and excretion. The ovarian follicular hormones—the estrogens—all possess an aromatic or benzenoid ring A, while the hormones of the testis, corpus luteum, and adrenal cortex are all characterized by the presence in ring A of an α-β unsaturated ketonic grouping, the saturation of which leads to practically complete loss of physiological activity. In the course of this reduction, which is a common mechanism of inactivation *in vivo*, carbon atoms numbers 3 and 5 become asymmetric, and hence four fully reduced geometrical isomers are possible in each instance. The hydroxyl group resulting on hydrogenation of the ketone may be oriented either *cis* or *trans* with respect to the angular methyl group at C_{10}. Respectively, the *cis* and *trans* epimers are designated by the suffixes β and α, and conventionally are differentiated in two-dimensional formulas by the use of a solid and a dotted line. [Compare androsterone (III) and isoandrosterone (IV).] 3(β)-Hydroxy-steroids form with digitonin (a saponin from *Digitalis*) sparingly soluble addition compounds, whereas the epimeric 3(α)-compounds do not. Likewise the hydrogen atom introduced at C_5 on saturation of a 4:5 or 5:6 double bond may be *cis* or *trans* oriented in relation to the angular methyl group at C_{10}; again, in planar formulation, the solid and dotted lines respectively are used [compare androsterone (III) and etiocholanolone (V)]. Because of this isomerism about C_5, there are two saturated parent hydrocarbons of the steroid hormones containing 19 carbon atoms, namely androstane (*trans*) and etiocholane (*cis*), and two pertaining to the hormones with 21 carbon atoms, namely pregnane (*cis*) and allopregnane (*trans*).

The total synthesis of steroids has been realized through a long sequence of intricate chemical reactions which, however, are not yet applicable commercially. Steroid hormones are prepared by degradation of naturally occurring plant and animal steroids. Diosgenin, a sapogenin from the Mexican yam, is readily converted to progesterone (XVIII) and deoxycorticosterone (XIV), and from these to testosterone (II) and estrone (VIII). Much less satisfactorily, the same four hormones may be derived from cholesterol. The bile acids, particularly deoxycholic acid, serve as starting material for the part-synthesis of the corticoids bearing an oxygen function at C_{11}, namely cortisone (XV), corticosterone (XVI), and Compound F (XVII). Certain microorganisms possess the unique and specific property of introducing a hydroxyl group at C_{11}, and they are employed in some commercial procedures for the preparation of cortisone. Isotope studies show that in the body all the steroid hormones and cholesterol can be synthesized from acetate.

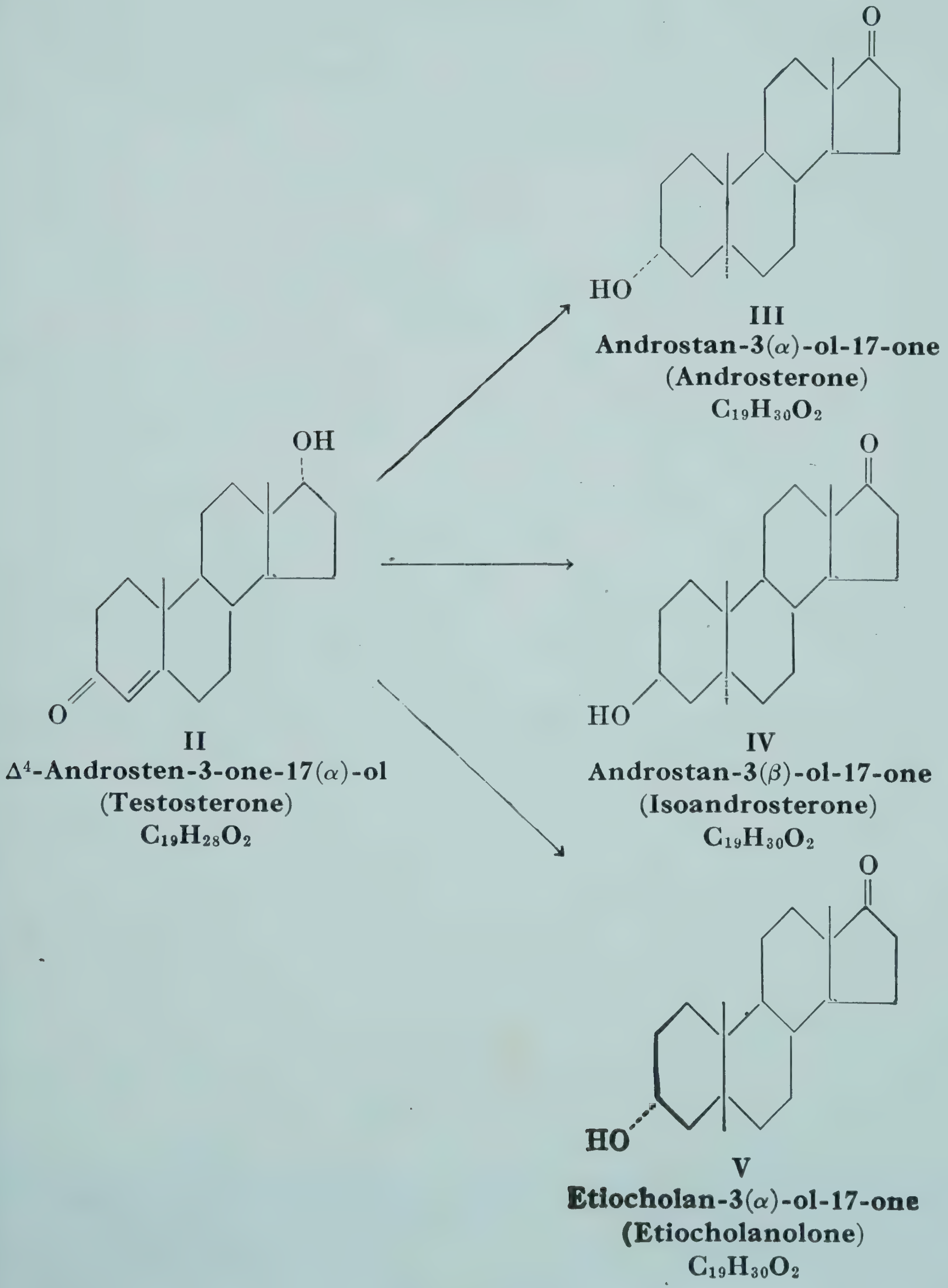
In general, all steroid hormones are insoluble in water but soluble in fat solvents such as ether, acetone, alcohol, etc., and in vegetable oils, in which medium they are usually dissolved for administration to the body. They are relatively inactive *per os*, and consequently are given intramus-


ANTERIOR PITUITARY
THYROTROPIC
PROLACTIN
ADRENOCORTICOTROPIC (ACTH)
GONADOTROPIC
GROWTH
THYROID
Thyroxine
Metabolic Rate
Luteotropic Action
Luteinizing Hormone (LH)
Follicle-Stimulating Hormone (FSH)
OVARY
Follicle
Corpus Luteum
ADRENAL CORTEX
TESTIS
Seminiferous Tubules
Interstitial Cells
Progesterone
Estrogens β-estradiol
Estrogens Progesterone
Mineralocorticoids S
Glucocorticoids E,F
Androgens
Androgens Testosterone
Uterus and Mammary Glands
Female Sex Characters
Electrolyte and Water Balance
Proteins Catabolism
Proteins Anabolism
Male Sex Characters

cularly or subcutaneously. Esters of the hormones, such as the acetate, propionate, and benzoate, are much more valuable therapeutically than the free steroids, since their physiological action is more protracted owing to the delayed rate of absorption and utilization. The steroid hormones and their catabolites are generally eliminated in the urine in water-soluble form in conjugation with glucuronic or sulfuric acid.

Formulation of Steroid Hormones and Related Substances. The chemical name is given in each instance, followed by the commonly used name in parentheses. The arrows indicate metabolic pathways followed in the body.

The Androgens and Urinary 17-Ketosteroids

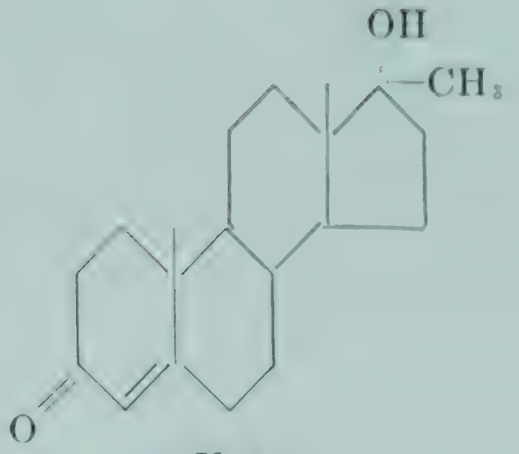


II
Δ^4-Androsten-3-one-17(α)-ol
(Testosterone)
$C_{19}H_{28}O_2$

III
Androstan-3(α)-ol-17-one
(Androsterone)
$C_{19}H_{30}O_2$

IV
Androstan-3(β)-ol-17-one
(Isoandrosterone)
$C_{19}H_{30}O_2$

V
Etiocholan-3(α)-ol-17-one
(Etiocholanolone)
$C_{19}H_{30}O_2$

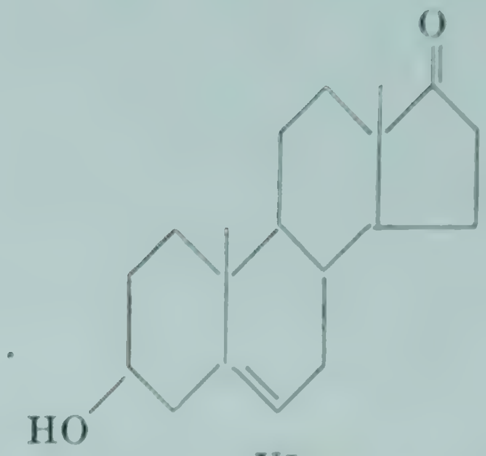


VII

17(β)-Methyl-Δ^4-androsten-3-one-17(α)-ol

(Methyltestosterone)

$C_{20}H_{30}O_2$



VI

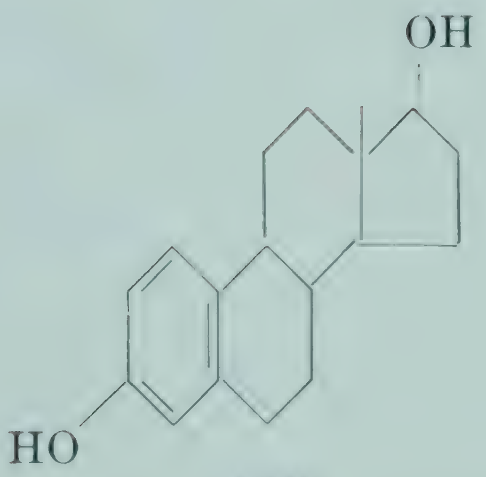
Δ^5-Androsten-3(β)-ol-17-one

(Dehydroisoandrosterone)

(*Trans*dehydroandrosterone)

$C_{19}H_{28}O_2$

The Estrogens



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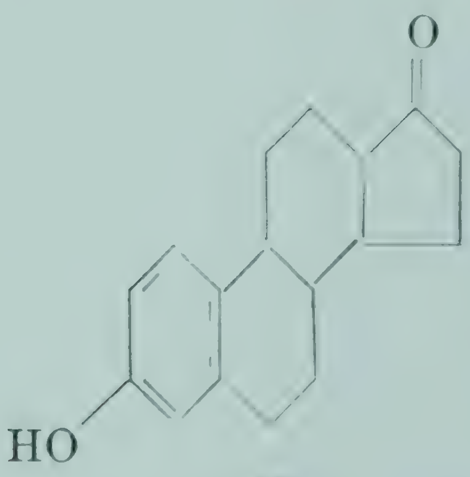


VIII

$\Delta^{1,3,5}$-Estratriene-3,17(α)-diol

(β-Estradiol)

$C_{18}H_{24}O_2$

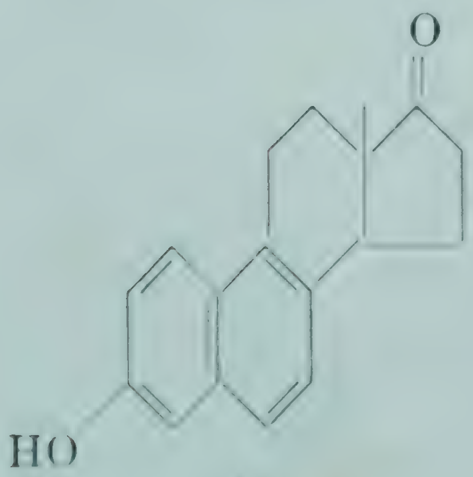
IX

$\Delta^{1,3,5}$-Estratriene-3-ol-17-one

(Estrone)

$C_{18}H_{22}O_2$

↓

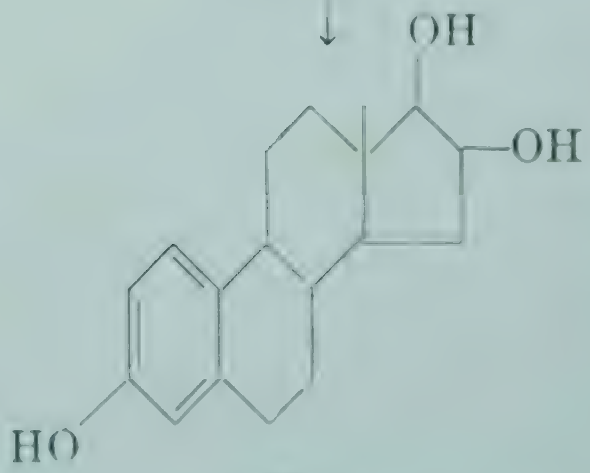


XI

$\Delta^{1,3,5;10,6,8}$-Estrapentaen-3-ol-17-one

(Equilenin)

$C_{18}H_{18}O_2$

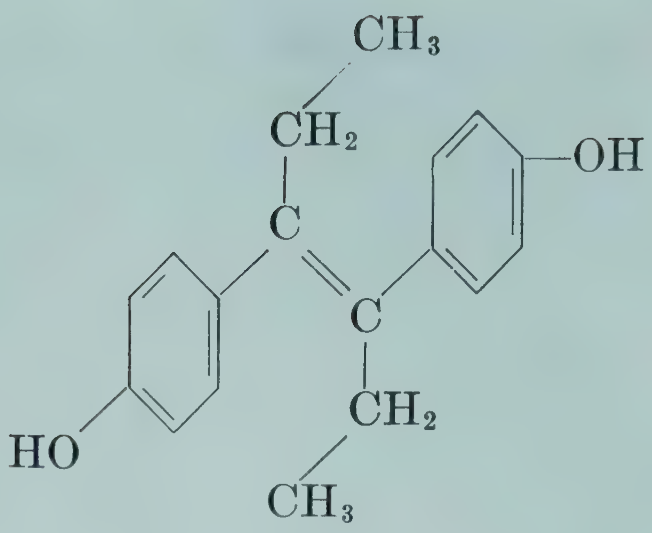


X

$\Delta^{1,3,5}$-Estratriene-3,16,17-triol

(Estriol)

$C_{18}H_{24}O_3$



XII

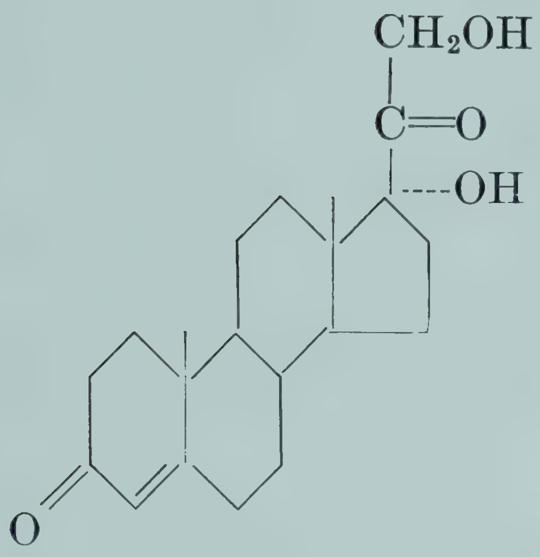
***trans*-3,4(Di-*p*-hydroxyphenyl)-hexene-3**

(Diethyl stilbestrol)

$C_{18}H_{20}O_2$

Adrenal Cortical Steroids

Mineralocorticoids



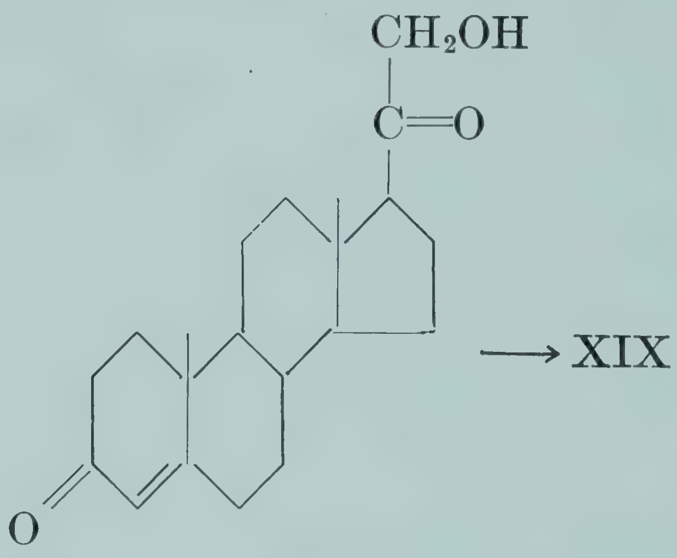
XIII

Δ^4-Pregnene-3,20-dione-17(α),21-diol

(17-Hydroxydeoxycorticosterone)

(Compound S)

$C_{21}H_{30}O_4$



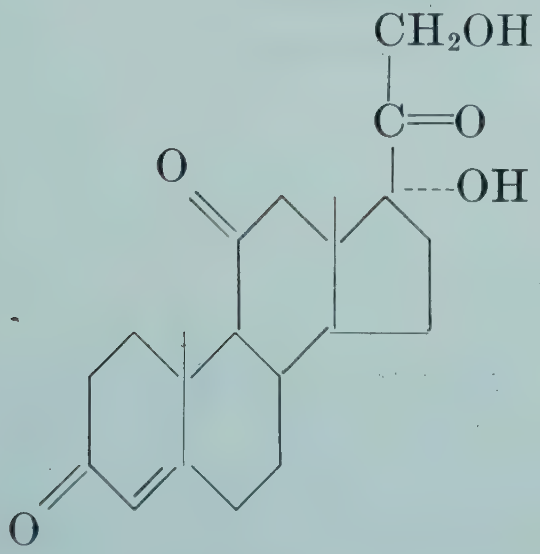
XIV

Δ^4-Pregnene-3,20-dione-21-ol

(Deoxycorticosterone)

$C_{21}H_{30}O_3$

Glucocorticoids



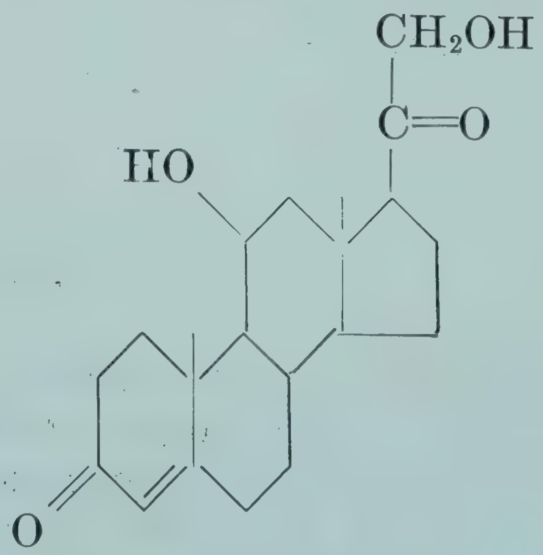
XV

Δ^4-Pregnene-3,11,20-trione-17(α),21-diol

(17-Hydroxy-11-dehydrocorticosterone)

(Cortisone, compound E)

$C_{21}H_{28}O_5$

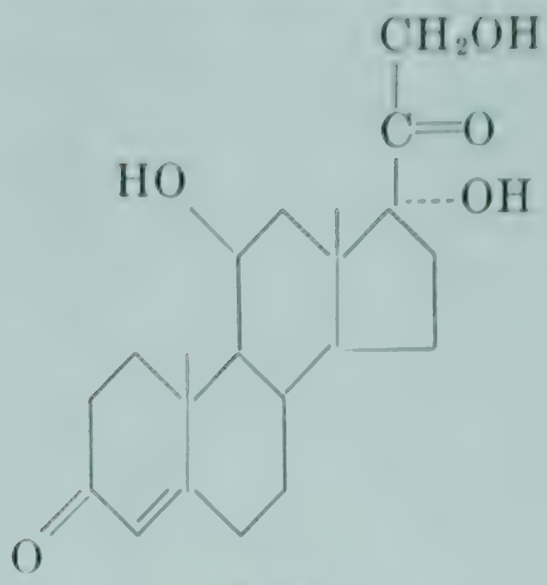


XVI

Δ^4-Pregnene-3,20-dione-11,20-diol

(Corticosterone, compound B)

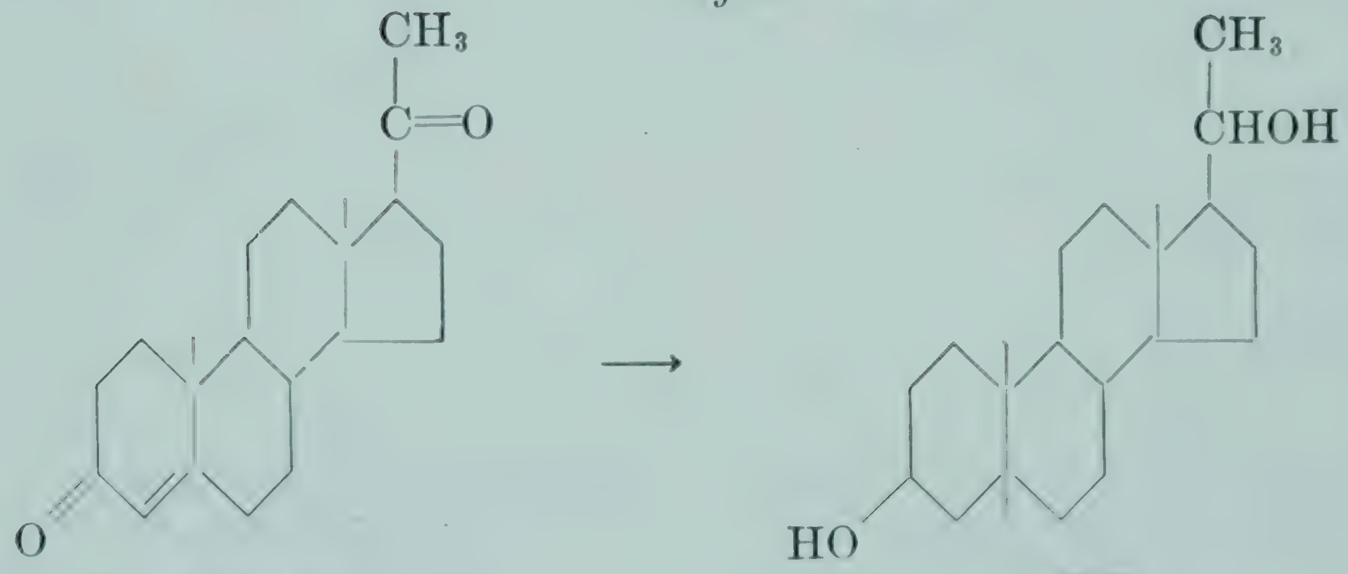
$C_{21}H_{30}O_4$



XVII

Δ^4-Pregnene-3,20-dione-11(β),17(α),21-triol
(11-Dihydrocortisone, 17-Hydroxycorticosterone)
(Compound F)
$C_{21}H_{30}O_5$

Progesterone



XVIII
Δ^4-Pregnene-3,20-dione
(Progesterone)
$C_{21}H_{30}O_2$

XIX
Pregnane-3(α),20(α)-diol
(Pregnanediol)
$C_{21}H_{36}O_2$

THE TESTES

Removal of the testes causes atrophy of the seminal vesicles, prostate, and other accessory organs of the male genital system. The effects are most pronounced if the operation is performed before sexual maturity, and are due to withdrawal of androgenic hormones, such as testosterone (II), secreted by the Leydig or interstitial cells. The androgenic hormones will restore the atrophied tissues in castrated animals, such as rats or mice, and are frequently assayed by their power to cause enlargement of the comb in the capon.

Testosterone (II) is the principal male sex hormone isolated from testis tissue. In the course of its metabolism in man it is reduced to androsterone (III), isoandrosterone (IV) and etiocholanolone (V), all of which are excreted in the urine (see 17-ketosteroids, p. 759). Methyltestosterone (VII), prepared artificially, but not known to occur in nature, finds wide therapeutic application because it is highly active *per os.*

The International Standard male hormone unit is the activity equivalent of 0.1 mg. of pure androsterone. By comparison of comb growth in the capon, testosterone is about six times, and isoandrosterone one-seventh, as potent as androsterone, while etiocholanolone is practically inert. Administered parenterally, methyltestosterone and testosterone show equal activity; orally methyltestosterone is two to four times less active than parenterally, but about 20 times more potent than testosterone given by mouth.

THE OVARIES

The development of the accessory sex organs in the female is not so dependent on hormonal stimuli as in the male; nevertheless, extirpation of the ovaries causes atrophic change in the uterus, vagina, mammary glands, etc., and in the plumage of poultry. In most mammals, mating occurs only during periods of heat or *estrus*, when the concentration of estrogenic hormones produced by the ovary and in particular by the Graafian follicles becomes maximal. In rodents estrus is accompanied by the appearance of cornified cells in the lumen of the vagina, from the walls of which they have desquamated. This reaction follows the administration of estrogenic substances to castrated animals, and is the basis of biological assay.

After ovulation in mammals, the ruptured Graafian follicle is transformed into the corpus luteum, which produces the hormone progesterone (XVIII) whose chief function is to prepare the mucous membrane of the uterus for the implantation of the embryo. Progesterone is assayed by its power to cause a glandular "progestational" proliferation of this membrane in immature or castrate rabbits previously sensitized with estrogens. Gestation, in its early stages, is interrupted by destruction of the corpora lutea; when implantation and pregnancy occur, the active life of the corpora lutea is prolonged. In the later stages of human pregnancy, estrogens and progesterone are produced by the placenta; they act together in promoting mammary development.

The principal estrogens are β-estradiol (VIII), estrone (IX), and estriol (X); their relative activities are roughly 10:1:1 or less, but wide fluctuation in these proportions is observed by the different methods of testing. Only the first two are known to occur in *liquor folliculi*, where β-estradiol accounts for 90 per cent of the physiological activity, and hence is regarded as the chief ovarian follicular hormone. β-Estradiol and estrone are interconvertible in the animal organism, for the administration of either leads to the excretion of the other. Estriol has been isolated only from human pregnancy urine and from human placenta, and that organ is regarded as the main site of production. In gestation (Fig. 202), the estrogen output rises sharply with the growth of the placenta to a prepartum level of 12 to 40 mg. *per diem;* estriol constitutes the chief catabolite (about 90 per cent of the total), but estrone is excreted in appreciable amount together with traces of β-estradiol. The conjugate of estriol in human pregnancy urine has been isolated as the 16- or 17-monoglucuronide. Throughout the normal menstrual cycle, the urine estrogen content is low, of the order of 0.08 mg. *per diem* at the maximum. Biological assay reveals pre- and postovulatory peaks with the minimum output during the period of flow. While the chemical nature of the estrogens excreted in the nonpregnant state has not been established by isolation, partition experiments indicate that all three are eliminated.

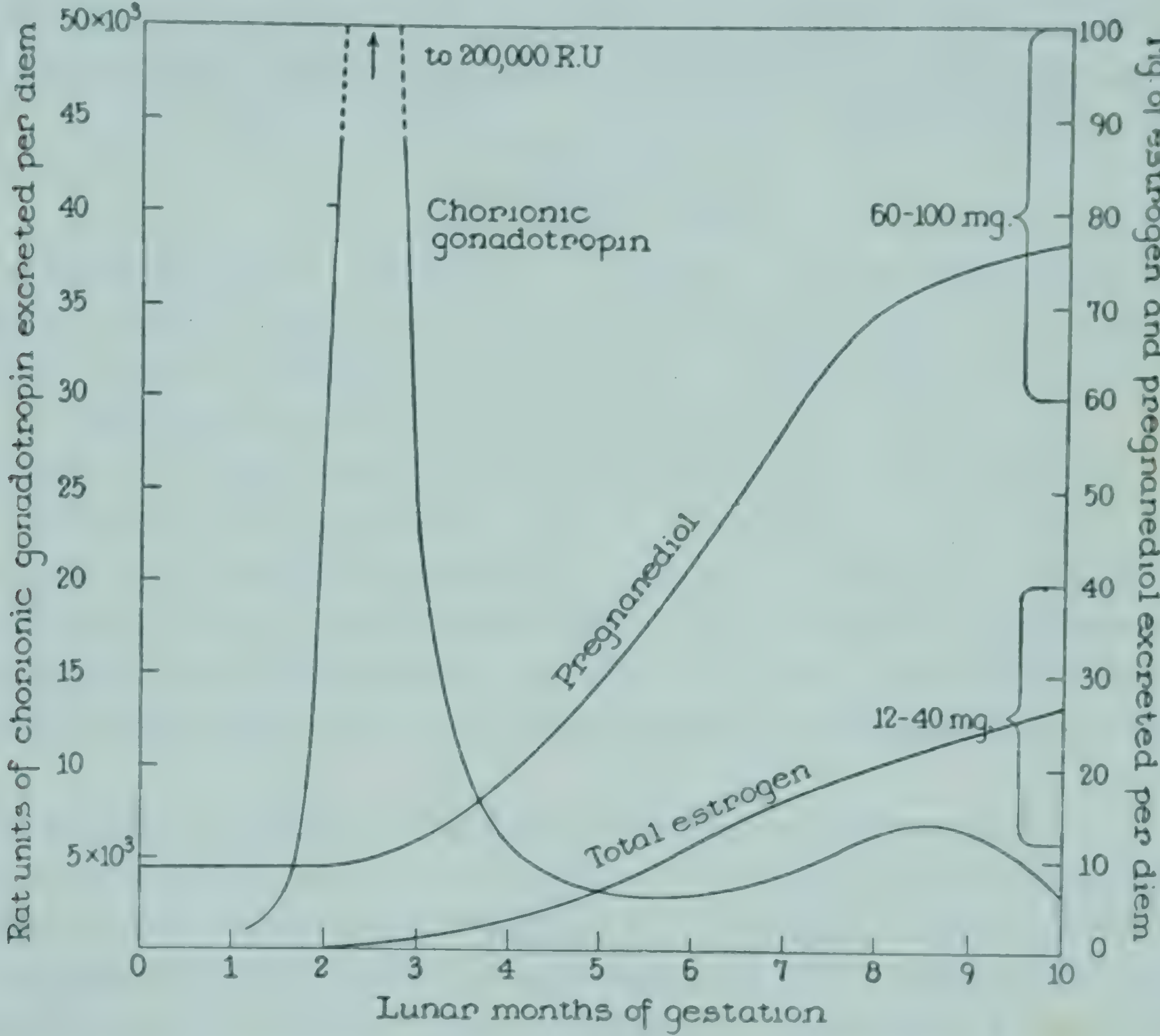


FIG. 202. VARIATION IN URINARY OUTPUT OF SEX HORMONES AND METABOLITES WITH PERIOD OF GESTATION.

Throughout gestation in the mare, estrone represents the most abundant excretory product. Estriol is not formed in this species, but considerable amounts of estrogens unsaturated in ring B as well as in ring A are eliminated; *equilenin* (XI) is the most abundant.

Many synthetic derivatives of stilbene possess marked estrogenic potency, although they are nonsteroidal and do not occur in nature. Important among these is *diethyl stilbestrol* (XII) which, like other members of the group, is much more active orally than the free natural estrogens. The water-soluble conjugated estrogens of human and equine pregnancy urine (principally estriol glucuronide and estrone sulfate respectively) are also effective by mouth.

The International Unit is defined as the activity contained in 0.1 μg. of estrone. As the different estrogens cannot properly be compared because of variations in the time and period of action, a subsidiary standard exists for β-estradiol-3-monobenzoate which is the activity equivalent of 0.1 μg. of that substance.

Progesterone is produced by the corpus luteum of the ovary during the postovulatory phase of the menstrual cycle, and in much larger amounts throughout gestation by both the corpus luteum and the placenta. Traces are elaborated by the adrenal cortex in both sexes. The chief metabolite of progesterone (XVIII) is the inactive pregnanediol (XIX), which is excreted in the urine in conjugation with glucuronic acid. Estimation of the sodium pregnanediol glucuronidate content of urine thus provides a useful index of luteal function. From 40 to 55 mg. of pregnanediol are eliminated during the normal menstrual cycle, the excretion beginning probably one to two days after ovulation and ceasing one to three days before the onset of uterine bleeding. A significant decrease in total output, or shortening of the period of excretion, indicates that progestation is not sufficiently well developed or is of too brief duration to support implantation. From fertilization to the end of the ovarian phase of pregnancy (ca. three to four lunar months), urine pregnanediol remains at the maximum level associated with the luteal phase of the cycle (ca. 5 to 10 mg. *per diem*), and then rapidly rises with the growth of the placenta to a daily output at parturition of about 60 to 100 mg. (Fig. 202). A significant fall during the first third of gestation indicates corpus luteum deficiency or failure of the placenta to develop at the normal rate, either of which threatens abortion. A residual pregnanediol titer of the order of 0.2 mg. *per diem* remains in the urine of men and of women in the follicular phase of the cycle; presumably this is of adrenal cortical origin. The International Unit is defined as the activity contained in 1 mg. of the pure crystalline progesterone.

THE ADRENAL CORTEX

The adrenal glands consist of two distinct tissues in close apposition: the *medulla*, producing epinephrine (adrenaline) and norepinephrine (noradrenaline) and derived from the sympathetic nervous system (see p. 766); and the much larger surrounding *cortex*, embryologically related to the gonads. Extirpation or destruction of the cortical tissue is fatal. Before death, the body loses sodium salts in the urine and accumulates potassium, so that the volume of extracellular fluid is reduced, the blood becomes viscous, the circulation sluggish, and renal function seriously impaired; treatment with diets low in potassium and enriched with sodium salts is helpful. Another group of symptoms includes diminished breakdown of tissue proteins to form glycogenic amino acids, so that glycogen stores decrease rapidly on fasting, hypoglycemia develops, and resistance is greatly lessened to extremes of heat and cold, infections, toxic drugs, trauma, fatigue, and stress of all kinds. Addison's disease, in which the cortical tissue is gradually destroyed, is characterized by asthenia, emaciation, low blood pressure, pigmentation of the skin, and often hypoglycemia, although here (as in the adrenalectomized dog) the disturbance of electrolyte metabolism is usually the greatest threat to survival.

About 30 steroids have been isolated in crystalline form from cortical extracts. Of these 30 steroids, many are physiologically quite inert; others, such as traces of estrone (IX) and progesterone (XVIII) have been discussed above; several, such as androstenedione, adrenosterone, and 17-hydroxyprogesterone, have definite androgenic activity; but the most interesting are six which are active in correcting the specific effects of adrenalectomy and may therefore be called *corticoids* (Selye). All six are derivatives of Δ^4-pregnene-3,20-dione-21-ol. The adrenal contains enzyme systems which promote the introduction of oxygen at positions 11, 17, and 21 of the steroid skeleton; for example, adrenal tissue, in perfusion or incubation experiments, will readily convert progesterone (XVIII) to compounds F (XVII), E (XV) and B (XVI). *Deoxycorticosterone* (XIV) is present in very small amounts, if at all, but as it is readily available commercially, it has been widely used in medicine, especially as its 21-acetate (DCA); it is highly potent in correcting the imbalance in the metabolism of sodium and potassium and hence in maintaining life in the adrenalectomized dog or in patients with Addison's disease, and may be called a *mineralocorticoid.* On the other hand, *cortisone* (17-hydroxy-11-dehydrocorticosterone, Kendall's "compound E") (XV), and *hydrocortisone* (17-hydroxycorticosterone, Kendall's "compound F") (XVII) may be called *glucocorticoids;* they have slight and variable action on sodium and potassium balance but profound influence on protein and carbohydrate metabolism, on which their medical uses depend. *Corticosterone* (XVI) and *11-dehydrocorticosterone* possess both glucocorticoid and mineralocorticoid activity (oxygen at position 11 is essential for the former), but not in any high degree. It must be emphasized that cortical extracts contain mineralocorticoids with life-maintaining activity. These compounds (the "amorphous fraction") have not yet been crystallized or identified and are much more water-soluble than the glucocorticoids. Biological assay procedures for glucocorticoids are based on their power to increase resistance to cold in adrenalectomized rats or to restore liver glycogen levels. Mineralocorticoids are assayed by their life-maintaining power or more specifically by their effect on the metabolism of sodium and potassium.

The structure and secretory activity of the adrenal cortex is controlled by the adrenocorticotropic hormone (ACTH) of the anterior pituitary. After hypophysectomy, the cortex atrophies, especially the inner layers, and low resistance to stress and impaired glycogen storage indicate that glucocorticoids are not being produced. The injection of a single dose of ACTH, in the rat, leads to rapid depletion of the cholesterol and ascorbic acid of the cortex (this is the basis of the Sayers assay method) with some temporary increase in cortical volume and various evidences of increased manufacture and discharge of corticoids, principally hydroxycorticosterone (compound F). The application of suitable stress, e.g., brief exposure to cold or the injection of a sublethal dose of epinephrine, will produce the same results by causing the pituitary to discharge ACTH. In human subjects the injection or discharge of ACTH or of glucocorticoids is evidenced by (*a*) a fall in the numbers of circulating eosinophils and lymphocytes, (*b*) an increased excretion of nitrogenous waste products, notably uric acid, and (*c*) increased urinary excretion of corticoids as determined by chemical or biological assay (but representing only a small fraction of the corticoids actually secreted or injected) and of 17-ketosteroids. ACTH and compounds E and F have proved dramatically successful in relieving the symptoms of rheumatoid arthritis and many other diseases; the disappearance of pain and the development of a sense of well-being are particularly remarkable. Prolonged treatment with small daily doses of cortisone appears to be both possible and effective. However excessive doses administered over a long period of time may induce symptoms of *Cushing's syndrome*, with tendencies toward obesity, hypertension, osteoporosis, and psychic disturbances. In patients disposed to diabetes, symptoms of this disease may be aggravated. Large doses are contraindicated in tuberculosis, gastric ulcer, and diabetes. Cushing's syndrome is thus attributed to overproduction of glucocorticoids. The concept of a causal relation between overproduction of mineralocorticoids and the *collagen diseases* (rheumatoid arthritis, periarteritis nodosa, etc.), attributed to Selye, has been questioned by many investigators and is the subject of active study. Overproduction of adrenal androgens, with high 17-ketosteroid excretion, leads to the adrenogenital syndrome (*virilism*, *hirsutism*, or precocious masculinization) with enhanced protein synthesis and muscularity.

17-KETOSTEROIDS

Human urines contain steroids carrying ketonic oxygen at C_{17}, some of which are phenolic while others are neutral. The latter are commonly called "17-ketosteroids." The principal members of this group are androsterone (III), etiocholanolone (V), isoandrosterone (IV), and dehydroisoandrosterone (VI); the first two of these arise in part from the testis, while all represent the excretion products of some of the steroids of the adrenal cortex. Accordingly, measurement of 17-ketosteroid output provides a biochemical index of testicular and adrenocortical activity.

The normal 17-ketosteroid excretion of men between the ages of 20 and 40 years averages about 15 mg. *per diem;* normal women in the same age group excrete approximately 10 mg. *per diem.* In children under 8 years of age, less than 1 mg. *per diem* is eliminated, but from this age on there is a gradual increase to adult values. Likewise in old age a significant diminution is observed. As gonadectomy in the male decreases the average output from 15 to 10 mg., and is without effect in the female, it is concluded that approximately 10 mg. are derived from the adrenal cortex and 5 mg. from the testis. The human ovary is not a source of neutral 17-ketosteroid.

Disorders of the testis, adrenal cortex, and anterior pituitary may profoundly alter 17-ketosteroid excretion. In eunuchoidism values from normal to that of the surgical castrate (10 mg.) are reported, whereas in the rare cases of masculinizing tumors of the interstitial cells of the testis the output may reach 800 mg. *per diem.* In Addison's disease in the male, excretion falls to 1.2 to 6.4 mg., which represents the testicular output, while in the female practically no 17-ketosteroids are produced. In those cases of Cushing's syndrome not associated with carcinoma of the adrenal cortex, normal or only slightly elevated 17-ketosteroid values are observed (10 to 36 mg.), but when carcinoma of the cortex complicates the condition a much higher excretion is usually encountered (40 to 288 mg.). Similarly, in the adrenogenital syndrome, simple hyperplasia of the cortex leads to only a moderately high 17-ketosteroid output (up to 100 mg. approximately), whereas carcinoma generally gives rise to a more marked increase (ca. 100 to 250 mg.). In both instances, carcinoma may be distinguishable from hyperplasia by the higher excretion, and also by the increased proportion of the 3(β)-hydroxy-17-ketosteroids (chiefly dehydroisoandrosterone) to the 3(α). Normally, and in hyperplasia, the $\beta:\alpha$ ratio is 1:9, whereas in carcinoma it may rise to about 1:1. In panhypopituitarism, a general underproduction of all anterior-lobe hormones, the 17-ketosteroid excretion is low (0 to 3 mg.).

EXPERIMENTS ON STEROID HORMONES[1]

1. Partition and Estimation of the Estrogens of Urine: Principle. The determination comprises (*a*) hydrolysis of the water-soluble conjugates in urine, (*b*) extraction of the steroid moieties and their separation by partition between solvents, and (*c*) estimation of the estrogen content of the various fractions either colorimetrically (see Exp. 3) or by biological assay (see Exp. 4). Colorimetric methods are unsatisfactory when the estrogen output is less than 1.5 mg. per liter approximately, as in normal urine and throughout the first four months of gestation.

Procedure: Method of Bachman and Pettit.[2] Acidify 100 ml. of human pregnancy urine to pH 2 (thymol blue) with HCl. Add 5 ml. concentrated HCl, and boil the mixture under reflux for one hour. The hydrolyzed urine is cooled and diluted with 100 ml. of distilled water, and extracted once with 200 and twice with 100 ml. of peroxide-free ether. The combined ether extracts are washed with 8 ml. of 9 per cent $NaHCO_3$ (anhydrous), which is discarded, and are evaporated to dryness. The residue is dissolved in the minimum volume of ethanol (not over 0.5 ml.), and diluted with 35 ml. of benzene.

Estriol and extraneous phenols and acids are now removed from the benzene solution by extraction once with 35 and twice with 17.5 ml. of 9 per cent Na_2CO_3 (anhydrous) and once with 3.5 ml. of water; these extracts are combined and acidified to a pH less than 6 with HCl. The estriol fraction is collected with ether (three extractions with 40 ml.), which is washed with 12 ml. of 9 per cent $NaHCO_3$ and taken to dryness. The residue is again taken into benzene (50 ml.) with the aid of ethanol (0.5 ml.) and further purified by washing with 9 per cent $NaHCO_3$ (1 ml.) and by transference to water (extraction three times with 50-ml. portions). Evaporation of the combined water extracts to dryness *in vacuo* yields Fraction T, which is more than 50 per cent estriol by weight.

The first benzene solution (35 ml.) contains the estrone and estradiol which, after washing of the benzene solution once with 10 ml. of 45 per cent H_2SO_4 (by volume) and twice with 20 ml. of water, are removed by extraction with N NaOH (four times with 35-ml. portions). The combined alkaline extracts are acidified with HCl and extracted once with 225 and twice with 125 ml. of benzene. The combined benzene extracts are concentrated to about 50 ml.,

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[1] Space does not permit incorporation of the many methods for the estimation of the steroid hormones and their metabolites in urine and blood. For full practical details, reference should be made to *Methods in Medical Research*, Vol. 2, Chicago, Year Book Publishers, 1950; or to Emmens: *Hormone Assay*, New York, Academic Press Inc., 1950.

[2] Bachman and Pettit: *J. Biol. Chem.*, **138**, 689 (1941).

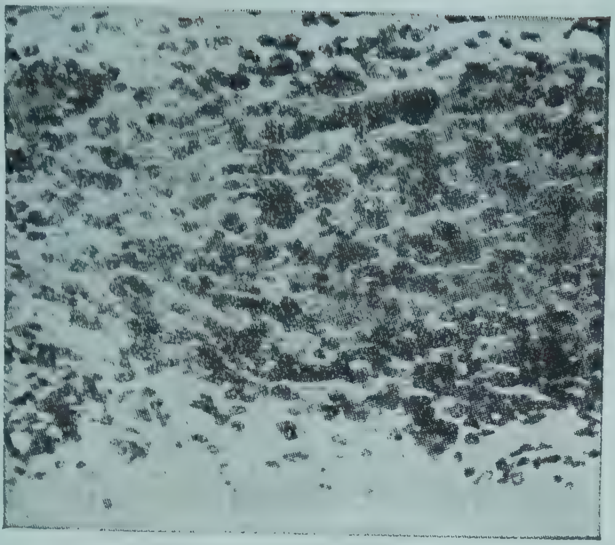

FIG. 203.

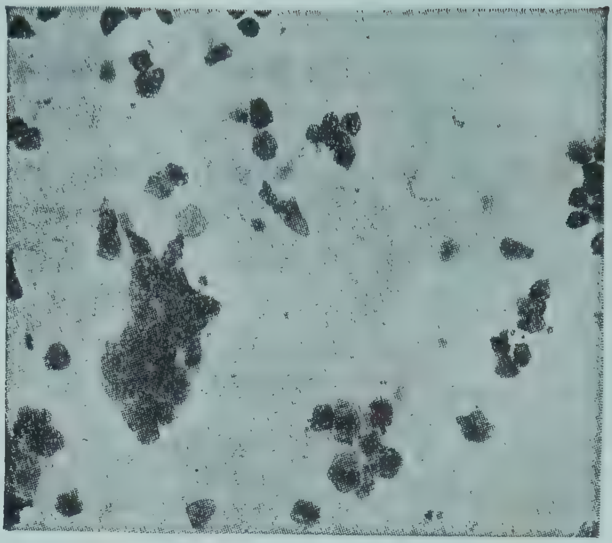

FIG. 204.

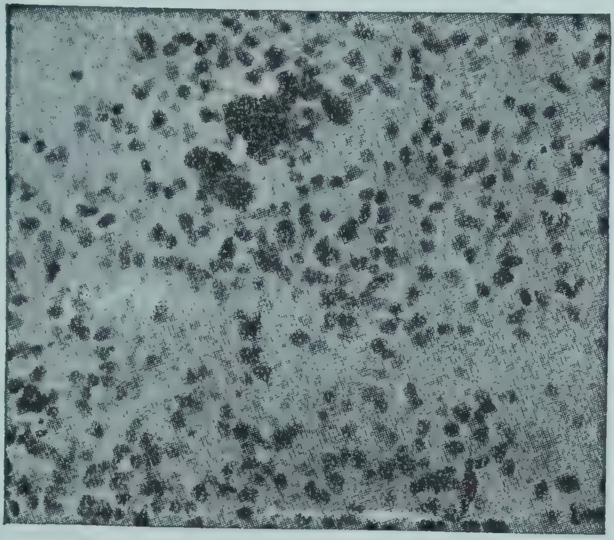

FIG. 205.

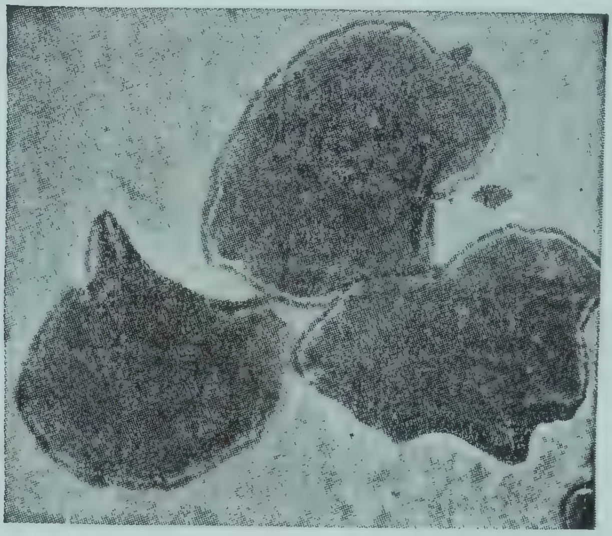

FIG. 206.

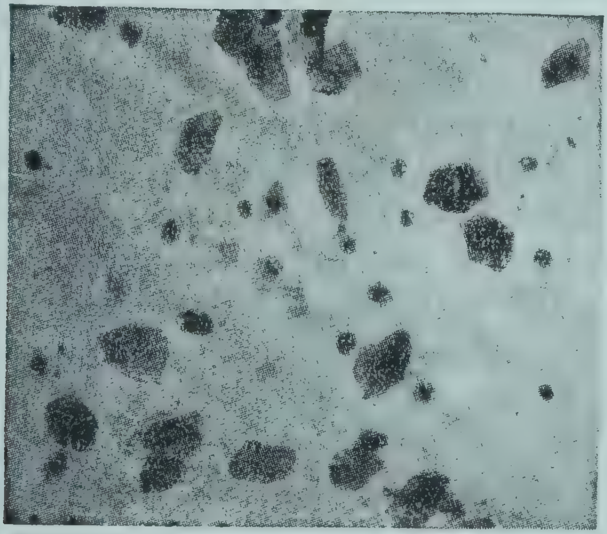

FIG. 207.

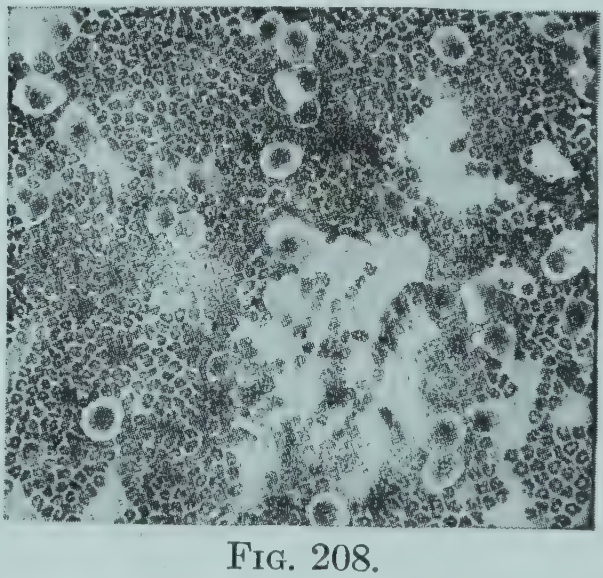

FIG. 208.

FIGS. 203–208. VAGINAL SMEARS OF ARTIFICIALLY INDUCED ESTRUS IN SPAYED RAT.

FIG. 203. Diestrous smear: leukocytes in stringy mass (× 40).

FIG. 204. Proestrous smear: chiefly nucleated epithelial cells with an occasional leukocyte (× 40). Present 35 to 40 hours after first injection.

FIG. 205. Estrous smear: nonnucleated cornified epithelial scales (× 40). This type usually appears within 48 hours after the first injection and is a certain criterion of the positive action of an extract.

FIG. 206. Flat, cornified elements of the estrous smear stage (× 250). Eosin stains these cells a brilliant red. Although the site of the former nucleus is apparent, all basophilic staining reaction has been lost.

FIG. 207. Early stage of leukocytic infiltration (metestrum) (× 40). Few nucleated epithelial cells have appeared as yet.

FIG. 208. Late stage of the metestrum (× 40). Enormous numbers of leukocytes, some cornified scales (in center of field), and many nucleated epithelial cells.

From Allen, Doisy, *et al.: Am. J. Anat.*, **34**, 169 (1924–1925). Courtesy, Cameron: *Recent Advances in Endocrinology*, 5th ed., Philadelphia, The Blakiston Company, 1945.

washed successively with 12 ml. of 45 per cent H_2SO_4, twice with 25 ml. of 9 per cent Na_2CO_3, and twice with 25 ml. of water, and evaporated to dryness (Fraction OD).

Both Fractions T and OD are purified sufficiently that they may be assayed colorimetrically (see Exp. 3). When a separate estimate of the estradiol and estrone content is required, Fraction OD is divided into ketonic (estrone) and nonketonic (estradiol) fractions by treatment with Girard's reagent[3] prior to assay.

2. *Isolation of Crystalline Estriol from Human Pregnancy Urine.* Fraction T (Exp. 1) from late pregnancy urine readily yields reasonably pure estriol on crystallization from benzene. For this purpose 500 ml. or more of urine may be taken, all quantities of reagents and solvents being increased proportionately.

3. *Kober's Color Reaction for Estrogens.*[4] The sample to be tested is evaporated to dryness in a test tube, to which is added 2 ml. of an equimolecular mixture of sulfuric and (*o* and *p*) phenylsulfonic acids. The test tube is heated for 10 minutes at 100° C. and at once cooled in ice. A red color gradually develops.

4. *Biological Test for Estrogens.*[5] Young adult female rats or mice are ovariectomized. If the operation is complete, within a week the vaginal smear will contain only leukocytes. The smear may be obtained by washing out the vagina with a drop of saline solution, using a small homemade pipet with a rubber bulb, or by introducing a small pledget of moist cotton wool on a loop of platinum wire, and in either case spreading the fluid on a clean microscope slide. It is not necessary to fix or stain the cells. Inject the animals with three 0.5 ml. doses of a solution of estrin in oil, or in 10 per cent alcohol made slightly alkaline, preferably allowing at least 6 hours to elapse between injections. Examine the vaginal smear on the second, third, and fourth days. The leukocytes will almost disappear, and the smear should come to consist of horny, scale-like squamous cells, and some epithelial cells still retaining their nuclei. The test can be made quantitative only if large numbers of animals are used, and their sensitivity to crystalline estrogens must be established. The response is affected by the strain of the animals, by the degree of subdivision of the dose and the solvent used, by the number of examinations made, and by many other factors. See Figs. 203–208.

5. *Determination of Sodium Pregnanediol Glucuronidate in Urine: Method of Venning.*[6] Urine is collected with four to five drops of tricresol as preservative and kept chilled until extraction to prevent hydrolysis. In the nonpregnant state a 48-hour specimen is required, in early pregnancy, a 24-hour specimen, and in late pregnancy, part thereof.

The urine is extracted 3 times with normal butanol (200, 100, 50 ml. per liter of urine). Gentle shaking (20 to 25 times) prevents formation of excessive emulsion. The combined butanol extracts are shaken vigorously, allowed to stand for 1 hour, when the urine which collects at the bottom is run off and the butanol is evaporated in a distilling flask almost to dryness under reduced

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[3] $(CH_3)_3 \cdot NCl \cdot CH_2 \cdot CO \cdot NH \cdot NH_2$. Girard and Sandulesco: *Helv. chim. acta*, **19**, 1095 (1936)

[4] Kober: *Biochem. Z.*, **239**, 209 (1931).

[5] Marrian and Parkes: *J. Physiol.*, **67**, 389 (1929).

[6] Venning: *J. Biol. Chem.*, **119**, 473 (1937) and **126**, 595 (1938).

pressure. The residue is taken up in 60 ml. of 0.1 N NaOH, again extracted 3 times with butanol (60, 20, and 10 ml.), and the combined butanol extracts are washed twice with 5 ml. of water and evaporated to dryness under reduced pressure. The residue is dissolved in exactly 5 ml. of water and transferred to a 125-ml. flask with several small portions of acetone. Volume is made to 100 ml. with acetone, and, after standing overnight in the refrigerator, pregnanediol glucuronidate precipitates. Most of the supernatant acetone is removed by suction without disturbing the precipitate. The remaining mixture is transferred to a 50-ml. centrifuge tube and centrifuged for 10 minutes. The residual acetone is carefully decanted and the crude ester is dissolved in water for purification by reprecipitation. The amount of water used is dependent upon the weight; 2 ml. for quantities less than 5 mg.; 3 ml. for 5 to 10 mg.; and 5 ml. for more than 10 mg. The water is added to the original flask to dissolve adhering material and then transferred to the 50-ml. centrifuge tube containing the precipitate. The tube is warmed and the contents are filtered back with suction into the original flask. The tube is rinsed twice with small amounts of acetone which are filtered into the flask, and the volume is made to 100 ml. with acetone. After 12 hours in the refrigerator, the acetone is removed as before by suction and centrifugation. The precipitate is dissolved in 10 ml. of hot 95 per cent ethanol and filtered with suction into a tared beaker (30 ml.). The flask, centrifuge tube, and filter paper are washed twice with hot ethanol. After evaporation of the solution to dryness on the bath, the weight of the precipitate is determined.

CALCULATION. The percentage recovery of the ester depends upon the amount present and the volume of water used in the second precipitation; accordingly a correction factor is applied (see the table below). Sodium pregnanediol glucuronidate contains 61.7 per cent pregnanediol.

$$\text{Mg. pregnanediol excreted } per\ diem = \frac{\text{Wt. of ppt.} \times 0.617 \times 24\text{ hr. volume} \times 100}{\text{Per cent recovery} \times \text{volume extracted}}$$

The melting point of the final precipitate should always be confirmed; sodium pregnanediol glucuronidate melts at 273° C. with evolution of gas.

Weight of precipitate in mg.	*Per cent recovery*		
	2 ml. H_2O	*3 ml. H_2O*	*5 ml. H_2O*
2	0–60*	. .	. .
3–4	50–67*	. .	. .
5–8	70–75*	79	. .
9–10	. .	82	75
11–12	. . .	85	78
13–15	. .	. .	81
16–18	. .	. .	83
19–25	. .	. .	85

* Approximate values.

6. Preparation of Adrenal Cortex Extract: Method of Cartland and Kuizenga.[7] Minced whole beef adrenals are thoroughly extracted with 99 per cent acetone (2.5 liters per kg.) and again with 80 per cent acetone. The extracts are combined, filtered, and concentrated under reduced pressure (below 45° C.) till the acetone is removed. The aqueous residue is twice extracted with petroleum ether, which removes inert fats and is discarded, and then twice extracted with ethylene dichloride (400 ml. per kg. gland each time), which extracts the hormone but not adrenaline nor phospholipides. The ethylene dichloride soluble fraction is chilled to −15° C., and ice and precipitated solids are filtered off. The ethylene dichloride is removed under reduced pressure and the residue dissolved in ethyl alcohol. This is mixed with an equal volume of petroleum ether; water is then added to make the alcohol 90 per cent; this causes some of the petroleum ether to separate as an upper layer, which is removed in a separatory funnel and discarded. Two further discarded petroleum ether fractions are obtained by diluting the alcohol further to 80 and finally 70 per cent. The alcohol is now removed under reduced pressure, below 45° C., and the resulting aqueous colloidal solution suitably diluted to give a final preparation of volume 1 ml. per 40 g. gland, with NaCl added to make 0.9 per cent, and containing 10 per cent ethyl alcohol as preservative. This final solution is filtered and sterilized by Berkefeld filtration, and preserved in sterile ampoules in the refrigerator. (For details of this method, see the original paper.)

7. Chemical Assays of Corticoids: Principles. Several colorimetric methods have been described all of which depend upon the C_{20}–C_{21} ketol grouping in the side chain but are independent of the functional groups in the ring structure. These are based upon (*a*) reduction of phosphomolybdic acid to molybdenum blue, (*b*) reduction of cupric ion to cuprous ion, and (*c*) oxidative cleavage with periodic acid of carbon atom 21, which is released as formaldehyde and estimated with chromotropic acid. The methods are applied to the full 24-hour output of urine and lead to titers of "reducing steroids" and "formaldehydogenic steroids." All the methods include in the estimates the active corticoids, plus much larger quantities of inactive metabolites which respond to these reactions but are physiologically inert. Consequently the values determined by chemical assay (about 1 to 2 mg. per day in normal individuals) are considerably higher than the glucocorticoid titer. (See footnote 1, p. 760.)

8. Biological Assay of Glucocorticoids and Determination of Urinary Cortin by Liver Glycogen Deposition: Method of Venning, Kazmin and Bell.[8] The method is applicable to crystalline corticoids or to urine extracts. The reference standard is cortisone (XV), the biological activity of 1 μg. of which is defined as one glycogenic unit in the assay of unknown extracts.

Procedure. For urines containing a normal or low titer, a 48-hour specimen is required. The urine is adjusted to pH 1 with HCl, and extracted 3–4 times with ethylene dichloride or chloroform. Any emulsions encountered can be broken by centrifuging. The clear extracts are evaporated almost to dryness under reduced pressure (water bath temperature not over 55° C.). The residue is taken up in 30 ml. of chloroform, and the solution is extracted 3 times with

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[7] Cartland and Kuizenga: *J. Biol. Chem.*, **116**, 57 (1936). See also Kuizenga, Wick, Ingle, Nelson, and Cartland: *J. Biol. Chem.*, **147**, 561 (1943).

[8] Venning, Kazmin, and Bell: *Endocrinology*, **38**, 79 (1946).

5 ml. of cold 0.1 N NaOH and 3 times with 5 ml. of water. These washings are back-extracted with chloroform, the chloroform extracts combined, and evaporated to 1–2 ml. for transfer to a test tube. Finally they are evaporated to dryness under a stream of nitrogen. The dry residue may be stored until ready for assay.

For the bioassay male white mice weighing 20–25 g. are used. Two days before adrenalectomy the mice are taken off a stock diet of "Purina" chow and put on the McCollum lactation diet,[9] which contains 26 per cent protein and 52 per cent carbohydrate. Removal of the adrenals is effected by the usual lumbar route. Following the operation, the mice are kept in a constant-temperature room or box at 76° F., and maintained on the McCollum diet supplemented with NaCl (0.9 per cent) and glucose (5 per cent) in the drinking water for the first postoperative day. Glucose is withdrawn thereafter; salt is retained in the diet throughout the test. Food is removed at 5 P.M. of the third postoperative day, and the mice starved until the following morning. Drinking water is removed on the fourth postoperative day, and beginning at 9:15 A.M. a total of 7 injections are given at 9:15, 10:00, 10:45, 11:30 A.M., 12:30, 1:30, and 2:30 P.M. The material to be tested is taken into solution containing 5 per cent glucose and 10 per cent alcohol. At each injection 0.20 ml. is given subcutaneously, so that each mouse receives a total of 1.4 ml. of extract containing 70 mg. of glucose. At 3:30 P.M. the mice are weighed and anesthetized with Sodium Amytal (0.2 ml. of 1.8 per cent solution). The livers are quickly removed and plunged into 4 ml. of hot 30 per cent KOH contained in a 15-ml. graduated centrifuge tube. The tubes are heated in a boiling water bath and frequently shaken, until all the tissue is in solution. The glycogen is precipitated by the addition of 1.2 volume of 95 per cent alcohol. The tubes are reheated until the mixture just begins to boil, cooled in an ice bath, and centrifuged. The supernatant liquid is poured off, and the tubes are allowed to drain. The sides of the tubes are washed down with 0.5 ml. of alcohol and again allowed to drain. Final traces of alcohol are expelled by heating the tubes for a few minutes in the hot water bath.

The glycogen is hydrolyzed as follows: After addition of 5 ml. of 1 N H_2SO_4, the tubes are placed in an autoclave and heated for 15 minutes at 15 lb. pressure. The glucose is determined by the method of Good *et al.*[10] or colorimetrically by the method of Nelson[11] (see p. 573). The glycogen is expressed in terms of mg. of liver glucose per 100 g. of mouse body weight.

Six to eight mice must be used for each assay. For normal male urine the equivalent of 6 hours of urine is administered to each mouse, whereas for normal female urine expected to be low in glycogenic activity, the equivalent of an 8-hour aliquot of urine is given each animal.

Values found by this method range from 0.04 to 0.085 mg. per 24 hours for normal men, and from 0.025 to 0.065 for women, calculated as cortisone.

9. Determination of the Neutral 17-Ketosteroids of Urine: Principle. The steroids of urine are first set free from their water-soluble conjugates by acid hydrolysis and extracted with ether. After removal of phenolic and acidic impurities, the 17-ketosteroid content of the neutral residue is ascertained by quantitative application of the Zimmermann reaction.[12] This comprises coupling of the reactive 17-ketone group of the compounds with *m*-dinitrobenzene to form colored complexes, the intensity of which in the green region of the spectrum (520 mμ) is measured in the photoelectric colorimeter and compared with the color developed by known amounts of a pure crystalline 17-ketosteroid.

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[9] Cited by Agate and Zwemer, *Am. J. Physiol.*, **111**, 1 (1935).

[10] Good, Kramer, and Somogyi: *J. Biol. Chem.*, **100**, 485 (1933).

[11] Nelson: *J. Biol. Chem.*, **153**, 375 (1944).

[12] Zimmermann: *Z. physiol. Chem.*, **233**, 257 (1935).

Nonspecific chromogens of urine cause some interference, but as these substances are mainly nonketonic and absorb light in the violet (420 mμ) as well as the green (520 mμ) region of the spectrum, their influence may be eliminated either by carrying out the color determination on the neutral ketonic fraction of urine, or by the application of a correction factor, which, in effect, subtracts the color component due to extraneous chromogens.

Procedure.[13] **A 24-hour specimen of urine is collected and measured. To a 100-ml. aliquot, 15 ml. of concentrated HCl are added. The mixture is refluxed for exactly 15 minutes, and then immediately chilled and extracted 4 times with 30-ml. portions of freshly redistilled ether. The combined ethers are washed 4 times with 2N NaOH (15-ml. portions) and then twice with water. On evaporation of the ether, the neutral residue is dissolved in 10 ml. ketone-free ethanol. In the development and measurement of the color, matched colorimeter tubes must be used. To the experimental tube are added, in order and with mixing after the addition of each, 0.2 ml. of the urine extract, 0.2 ml. of 2 per cent pure *m*-dinitrobenzene (prepared fresh) in ethanol, and 0.2 ml. of 5N aqueous KOH (purest electrolytic grade). A control tube is made up in the same way containing 0.2 ml. of ethanol in place of the urine extract. Both tubes are incubated in the dark at 25 ± 0.5° C. for exactly 105 minutes. Then 15 ml. of 80 per cent ethanol are added to each, and, after mixing, the color intensity in each is measured in a suitable photoelectric colorimeter. To correct for the slight color developed by the reagents alone, the control tube is first inserted in the instrument with the 520 mμ filter in position, and the instrument adjusted to 0 density (100 per cent transmittance). The control is now replaced by the experimental tube, the reading noted and referred to the calibration curve pertaining to pure androsterone to give the equivalent number of mg. of this substance in the sample.**

CALCULATION.

$$\text{Mg. 17-ketosteroids excreted } \textit{per diem} = \frac{\text{Mg. observed} \times \text{ml. 24-hour urine volume}}{2}$$

For the application of factors correcting for interfering chromogens, readings are taken at 420 mμ also. For further details and modifications see footnote 1, p. 760.

When differential estimates of the 3(α)- and 3(β)-hydroxy-17-ketosteroids are desired, their separation is effected with digitonin prior to assay.

NITROGEN-CONTAINING HORMONES

ADRENAL MEDULLA

This organ, which gives a specific staining reaction with dichromates ("chromaffin cells"), contains the active nitrogenous bases *epinephrine* or *adrenaline* (XX) and *norepinephrine* (*noradrenaline*, *arterenol*) (XXI). Both may also be prepared synthetically; the latter appears to be more abundant in the circulating blood.

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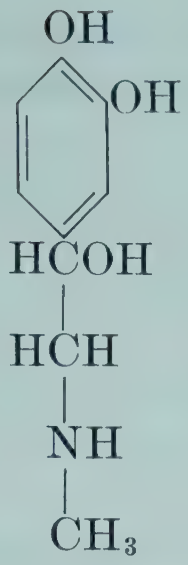
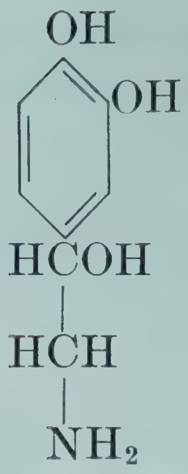
[13] Method used in the McGill University Clinic.



XX
Epinephrine



XXI
Norepinephrine

The natural forms are levorotatory and are many times more active than their optical isomers. They are basic substances, insoluble in weak alkalies and in organic solvents, and form water-soluble crystallizable salts with various acids. They are readily destroyed by oxidation in alkaline solutions, eventually giving rise to amorphous, dark-colored melanins. Being attacked both by "amine-oxidase" and by the cytochrome system they are quickly destroyed in the body, so that the effect of an intravenous injection rapidly passes off; they are slowly and incompletely absorbed from subcutaneous or intramuscular injection sites, where they are apt to produce abscesses; they are inactive orally though somewhat toxic. The physiological reaction to these hormones is, in almost all organs, the same as the response to stimulation of the sympathetic nerve supply: the blood pressure rises because of arteriolar constriction; the heart rate increases if the depressor reflex can be excluded; peristalsis is inhibited; the pupils dilate; the bronchial muscles relax; there is outpouring of ACTH and probably other anterior pituitary hormones; blood sugar and blood lactic acid increase at the expense of the glycogen stores; and the metabolic rate is temporarily increased. Norepinephrine shares the stimulatory rather than the inhibitory properties of the better-known epinephrine. Discharge of these hormones occurs only when stimuli reach the adrenal medulla by way of the splanchnic nerve. This happens rapidly when the body is exposed to emergencies such as cold or shock or violent emotion or pain.

EXPERIMENTS ON THE ADRENAL MEDULLA

1. *Preparation of Epinephrine.* To each 100 g. of ground fresh adrenal tissue add 50 ml. of a 3 per cent solution of acetic acid in 95 per cent ethyl alcohol. Allow to stand for 12 to 24 hours and remove the liquid by straining. Reextract the residue twice for 6 hours, each time with the same volume of 3 per cent acetic acid in 60 per cent ethyl alcohol as before. After filtering, concentrate the extract in a flask under diminished pressure to about one-tenth of the original volume. The material which has precipitated should be removed by filtration and the filtrate concentrated under diminished pressure to about 2 to 3 ml. per 100 g. of tissue extracted. Transfer the solution to a test tube and add enough strong ammonium hydroxide to leave a strong odor of ammonia. Stopper and set aside in a cool place for several hours. Remove the precipitate by filtration and wash first with ice-cold water which has been

boiled, then with cold alcohol, and finally with ether. Dissolve the precipitate in 10 ml. of 10 per cent hydrochloric acid, reprecipitate, and treat the precipitate as before.

2. *Properties of Epinephrine.* Dissolve 0.1 g. of epinephrine in about 10 ml. of 0.1 per cent HCl and dilute to 100 ml.

a. VULPIAN REACTION. **Add a few drops of $FeCl_3$ solution. A green color will be produced which is a typical catechol reaction.**

b. EWINS REACTION. **Add to 1 ml. of the solution an equal volume of 0.2 per cent potassium persulfate; a reddish color is produced which is specific and may be quantitatively determined.**[14]

c. FLUORESCENCE. **A faintly alkaline solution of epinephrine, even if extremely dilute, shows a bright apple-green fluorescence for some minutes when irradiated in a quartz vessel with ultraviolet light from a mercury vapor lamp.**

THE PANCREAS

Removal of the pancreas in cats and dogs produces symptoms similar to diabetes mellitus in man. Blood sugar increases greatly and glucosuria occurs; the glycogen stores of liver and muscle are depleted; the blood is charged with fat, and acetone bodies (acetoacetic acid, β-hydroxybutyric acid, and acetone), accumulate in the blood and are excreted in the urine, to produce acidosis, coma, and death, usually within three weeks. These symptoms do not follow when the digestive pancreatic juice is prevented from reaching the intestine, or even when the acinar cells which secrete this juice degenerate; they are rather due to removal of solid clumps of cells, the islets of Langerhans, which have no connection with the ducts but form a hormone, *insulin*, which is discharged into the blood and regulates carbohydrate metabolism. Diabetic symptoms appear when the islet cells are damaged by administration of alloxan, or in some species by prolonged administration of certain anterior-pituitary extracts which produce hyperglycemia.

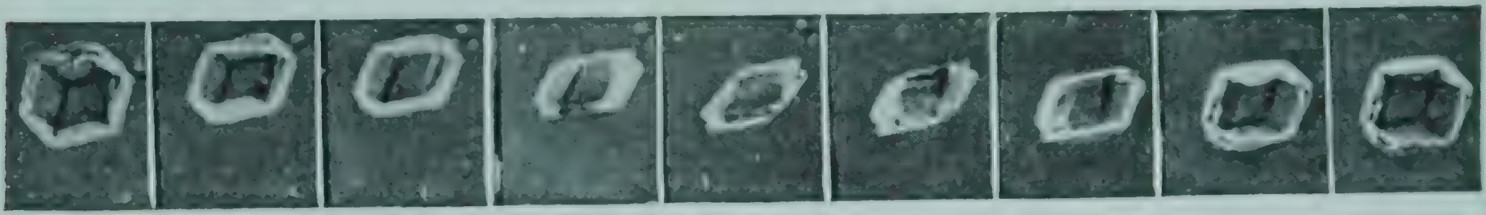


FIG. 209. CRYSTALLINE ZINC INSULIN.
Courtesy, Dr. D. A. Scott.

The first useful pancreatic extracts were prepared by extracting the pancreas with acid alcohol and increasing the concentration of alcohol in the filtered extracts until insulin was precipitated. Refinements of the method led to the isolation of insulin in crystalline form as a zinc salt (Fig. 209). Insulin is a protein somewhat of the albumin type, but unusually soluble in moderately dilute alcohol and acetone. It is destroyed by digestive enzymes and must be protected from pancreatic trypsin during extraction; hence, also, it is ineffective orally. Insoluble compounds of insulin with basic proteins (protamines or globin) and zinc are used therapeutically, since they are slowly absorbed from the tissues and injections need not be given so frequently. In normal animals, insulin lowers the blood sugar, thus eventually producing convulsions unless counteracted by the administration of glucose, or substance yielding glucose. The unit was at one time defined as one-third of the amount which will, in five hours, lower the blood sugar of a fasting rabbit to the convulsive level (45 mg. per 100 ml.), but assay is now conducted by comparing the activity of an unknown sample with that of crystalline insulin, either in lowering the blood sugar of rabbits or in inducing convulsions in mice: crystalline insulin is reckoned at 22 units per mg.

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[14] Barker, Eastland, and Evers: *Biochem. J.*, 26, 2129 (1932).

Some consider that insulin is an essential catalyst in the biological oxidation of carbohydrate; for example, that it favors the action of the enzyme hexokinase in forming glucose-6-phosphate as a first step; others believe that insulin checks the new formation of carbohydrate from fat (at least from glycerol) and protein, which in the absence of insulin is supposed to flood the organism with sugar; others again hold that lack of insulin inhibits reactions whereby a large proportion of dietary carbohydrate is converted into fat. The consequences of pancreatectomy are less marked in animals from which the anterior pituitary, or the adrenal cortices, are removed; they are in any case less marked in species other than the cat and dog.

Pancreatic extracts, including most insulin preparations, contain a *hyperglycemic factor* (HGF) which promotes the breakdown of liver glycogen; it has been suggested that this too is a hormone, produced by the α-cells of the islets, insulin almost certainly arising in the β-cells.

EXPERIMENTS ON THE PANCREAS

1. *Preparation of Insulin: Method of Jephcott.*[15] To 1 kg. or more of fresh, finely minced beef pancreas, add 4 volumes of extraction liquid, consisting of 750 ml. of ethyl alcohol, 250 ml. of distilled water, and 15 ml. of concentrated hydrochloric acid. Shake or stir for 2 hours at 37° C. and filter through a double layer of cheesecloth; extract the residues as before. Combine the filtrates and add concentrated ammonia till alkaline to litmus; centrifuge, and discard the precipitate. (Insulin may be precipitated quantitatively by adding 1½ volumes of absolute ethyl alcohol and 2½ volumes of ether and allowing to stand in the cold.) A purer preparation is obtainable as follows: After centrifuging, drive off the alcohol with a blast of air at 37° C. and add 40 g. of ammonium sulfate per 100 ml. The precipitate which rises to the top contains the insulin; it is ground with 70 per cent alcohol and filtered. To the filtrate add an equal volume of 95 per cent alcohol, and discard any precipitate which forms; now add 8 volumes of 95 per cent alcohol to precipitate the insulin, which is filtered off and dried, and dissolved in water (1 ml. per 10 g. of pancreas) containing 0.1 per cent tricresol.

2. *Effect of Insulin on Blood Sugar.* Take a 1-ml. sample of blood for a sugar determination from the marginal ear vein of a 2-kg. rabbit which has been starved for 24 hours. Then inject subcutaneously 5 to 10 units of insulin. Observe the rabbit carefully for symptoms of hypoglycemia, such as hyperirritability, palpitation of the heart, convulsions, and coma. Another sample of blood should be drawn as soon as distress is evident and a third when the rabbit is in convulsions. To relieve the induced hypoglycemia, inject 10 ml.

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[15] Jephcott: *Tr. Roy. Soc. Canada*, sec. 5, 25, 183 (1931).

of 10 per cent glucose solution intravenously. After recovery, take another blood sample for a sugar determination. Compare the blood sugar values. It is also possible to save the animal by injecting 0.5 ml. of 1:1000 epinephrine (adrenaline) subcutaneously after the first convulsions; or convulsions may be averted by feeding glucose upon a leaf of lettuce, or by giving glucose by stomach tube or intraperitoneally.

THE THYROID

The thyroid gland consists of a framework of connective tissue enclosing numerous vesicles lined with epithelium, flattened in the resting state and cubical or columnar (almost obliterating the colloid-filled cavity of the vesicle) when highly active. Marked enlargement of the gland is known as *goiter*. It may be associated with normal or subnormal activity, in which case it is related to a deficient intake of iodine and is chiefly found in inland regions, for example around the Great Lakes, where the soil, water, and vegetation are iodine-poor. It may also be associated with increased thyroid activity (Graves's disease, exophthalmic goiter). The thyroid is far richer in iodine than any other tissue. The iodine is chiefly built into the characteristic protein *thyroglobulin*, which makes up a large part of the colloid in the vesicles and may be isolated in relatively pure form.[16] Thyroglobulin on hydrolysis yields *thyroxine*,

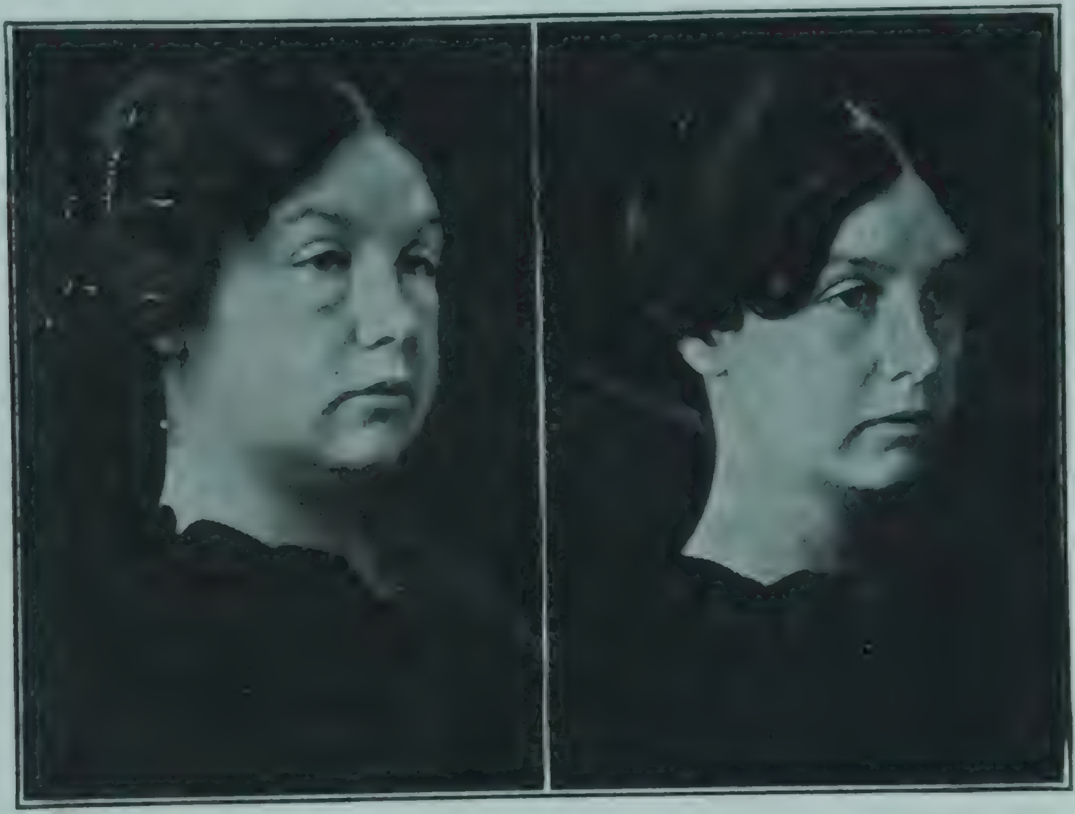
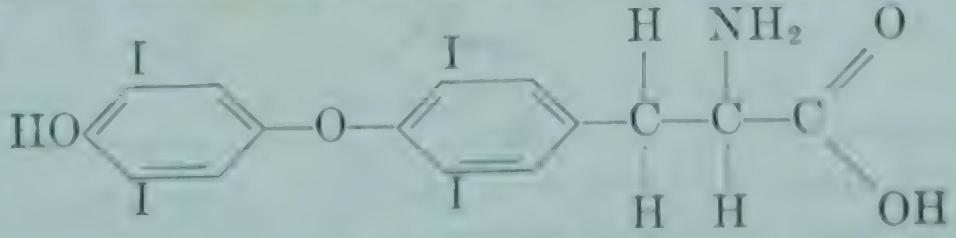


FIG. 210. EFFECT OF THYROXINE ON MYXEDEMA.
The time interval between pictures is three weeks. The total amount of thyroxine used was less than 20 mg.
From Kendall: *Ind. Eng. Chem.*, 17, 525 (1925).



Thyroxine

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[16] Heidelberger and Palmer: *J. Biol. Chem.*, **101**, 433 (1933).

or β-[3:5-diiodo-4-(3′:5′-diiodo-4′-hydroxy-phenoxy)-phenyl]-α-amino-propionic acid. This is usually obtained in the racemic form, but is originally levorotatory. Triiodothyronine, containing one iodine atom less, is also present and highly active. The administration of thyroxine or of substances containing it produces only in part the picture of Graves's disease: high metabolic rate, increased pulmonary ventilation and circulation rate tending to overwork the heart, extreme nervous restlessness, and sometimes protrusion of the eyeballs (exophthalmos). Deficient thyroid activity produces myxedema (see Fig. 210) with low basal metabolism, mental and physical sluggishness, and formation of a curious puffy tissue under the skin. The extreme form, in which severe deficiency dates from infancy, is known as *cretinism* (see Fig. 211), and is marked by stunting, deformity, and feeblemindedness. The actual hormone discharged by the gland is thyroxine, which appears to act as a stimulant of all metabolic processes; its effect is slowly developed and long-lasting, and it is not in general possible to discern it by applying thyroxine to isolated tissues or organs. Thyroxine is too insoluble to be given advantageously by mouth; but it is not destroyed during digestion, so that preparations of desiccated thyroid gland, given orally, are cheap and fully active. Methods of biological assay depend chiefly on measurements of the metabolism of myxedema patients, or of small animals; less accurate methods depend on the fact that thyroid substance accelerates the metamorphosis of tadpoles. Thyroxine may also be determined chemically by its iodine content, but it must be remembered that thyro-globulin also contains the more soluble, physiologically inactive amino acid:

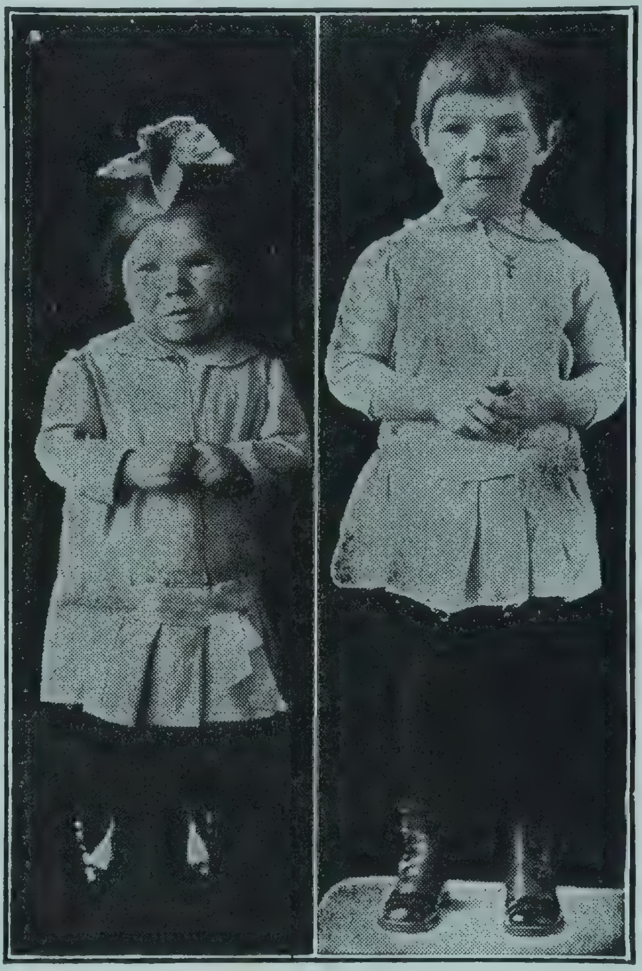
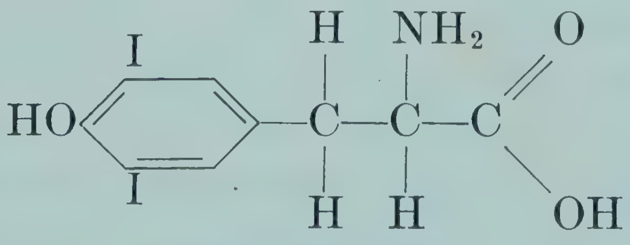


FIG. 211. EFFECT OF THYROXINE ON GROWTH OF A CRETIN.

The illustration shows the same child, in the same dress, before and after receiving thyroxine for one year. Increase in height, 6 inches.

From Kendall: *Ind. Eng. Chem.*, **17**, 525 (1925).



Diiodotyrosine

Experiments with the radioactive isotopes of iodine indicate that the gland, unless previously saturated, takes up iodide from the blood for the synthesis of diiodotyrosine and thence of thyroxine. These reactions can be inhibited by treatment with large doses of sulfa drugs or with thiourea, thiouracil, and related compounds, which thus gradually produce signs of hypothyroidism accompanied by goiter. Thiouracil is being used experimentally in the treatment of Graves's disease; the administration of iodine also produces a remission, which though often temporary is valuable in preparation for operation, and is due to retention of colloid within the gland.

The uptake of radioactive iodide (in the form of NaI or KI) can be followed by placing "counters" on the skin above the thyroid, and this has been used as a test of thyroid activity. In larger doses radioactive iodides have been used to control thyroid carcinoma and its metastases, since the intensity of radioactivity becomes much higher in the thyroid cells, normal or cancerous, than elsewhere in the body.

THE PARATHYROIDS

The parathyroid glands are small, compact bodies, usually four in number and closely apposed to or within the thyroid. If they are removed, the level of calcium in the blood serum falls; this in turn causes neuromuscular hyperexcitability, tremors of the skeletal muscle, and panting which produces alkalosis. With calcium deficiency, this leads to violent tonic and clonic convulsions in which the animal sooner or later dies from arrest of respiration, symptoms best seen in the dog. Acid extraction of ox parathyroids yields extracts which maintain parathyroidectomized dogs alive and free from tetany, and which in normal dogs produce an elevation of serum calcium. The (Hanson) unit is 0.01 × the amount required to raise the serum calcium of normal, 20-kg. dogs by 1 mg. per 100 ml., though larger increases can be more accurately measured. A large single injection may accelerate the renal excretion of phosphates and raise the serum calcium to twice its normal level in 12 to 15 hours, usually without disturbance to the health of the animal; but if the calcium is maintained at a high level by repeated injections, the blood becomes concentrated and the circulation sluggish, kidney function fails, and hemorrhage occurs in the gastrointestinal tract. The calcium mobilized by the parathyroid hormone comes from the skeleton, where it is probable that the hormone stimulates the formation and activity of the osteoclasts. After prolonged treatment or in the presence of a parathyroid tumor, the bones are demineralized and become fibrous and cystic (hyperparathyroidism: osteitis fibrosa). There is some tendency for the body to lose its responsiveness to parathyroid extracts; this is particularly well seen in the rat. The extracts being obtained by rather drastic procedures, there is no certainty that the principle they contain is the unaltered natural hormone; it is of protein nature[17] and destroyed by digestive enzymes.

EXPERIMENTS ON THE PARATHYROIDS

1. *Preparation of Parathyroid Extract: Method of Collip.* Place the fresh ox parathyroids, from which the visible fat and connective tissue have been removed, in a small flask or test tube, cover with an equal volume of 5 per cent hydrochloric acid, and keep in a boiling water bath for 1 hour. Allow to cool, and remove the congealed fat mechanically. Then make the solu-

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[17] Ross and Wood: *J. Biol. Chem.*, **146**, 49 (1943).

tion faintly alkaline with sodium hydroxide and add acid to the point of maximum precipitation of protein, which is just barely acid to litmus. Filter and dissolve the precipitate in weak alkali, and reprecipitate as before. Filter again and combine the filtrates, adding 0.1 per cent tricresol as preservative and keep the combined filtrates, which constitute the desired extract, in an icebox until ready to use.

2. *Effect of Parathyroid Extract.* Draw 8 to 10 ml. of blood from the saphenous or other suitable vein of a dog weighing at least 10 kg. Save this for determination of serum calcium (p. 644). Inject subcutaneously, every 3 hours for the next 9 to 12 hours, 10 Hanson units of commercial parathyroid extract (or a quantity of extract equivalent to 1 ox parathyroid) per kg. of body weight. Draw blood samples after the last injection, and again 12 hours later. Note the occurrence of depression and anorexia, the increased concentration of the blood, and the rise in serum calcium.

THE ANTERIOR PITUITARY OR HYPOPHYSIS

The anatomy of the pituitary gland, or hypophysis cerebri (Fig. 212) is complex: the *pars nervosa* is a downgrowth from the hypothalamic region of the brain, and consists of modified nervous and glial cells, while the rest of the organ is derived from Rathke's pouch, an upgrowth from the roof of the mouth. This gives rise to the *pars tuberalis,* which surrounds the stalk of the pars nervosa like a collar and spreads along the base of the brain, to the *pars intermedia,* closely apposed to the anterior surface of the nervosa and usually separated by a cleft (the cavity of the embryonic pouch) from the large, glandular *pars anterior* which lies in front and on either side of it. The gland can be broken in two at the cleft, leaving the *intermedia* and *tuberalis* adherent to the nervosa as a complex called the posterior lobe. The whole organ rests in a more or less fitting cavity in the sphenoid bone, the *sella turcica.*

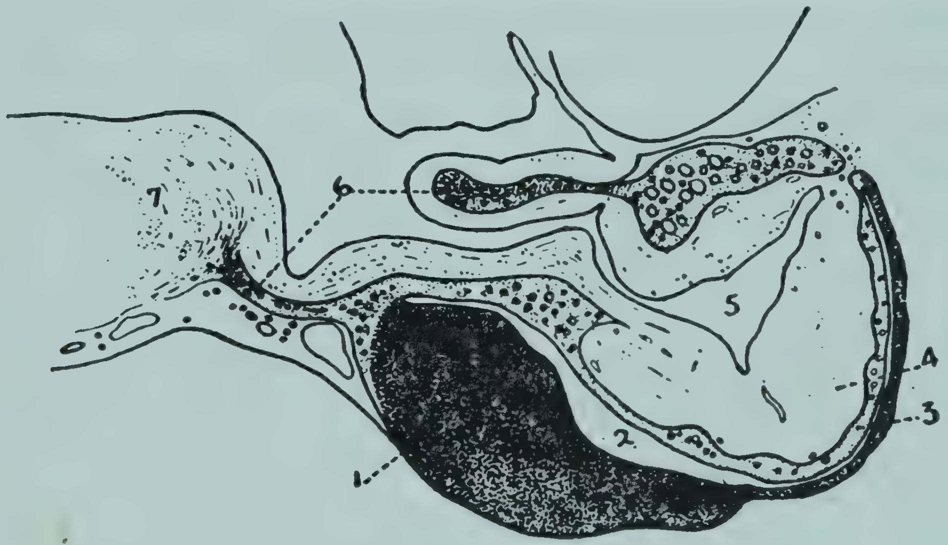


FIG. 212. HYPOPHYSIS OF CAT.

1, Pars anterior; *2,* hypophysial cleft; *3,* pars intermedia; *4,* pars nervosa; *5,* infundibular cavity; *6,* pars tuberalis; *7,* optic chiasm.

From Best and Taylor: *Physiological Basis of Medical Practice.* Courtesy, William Wood & Co.

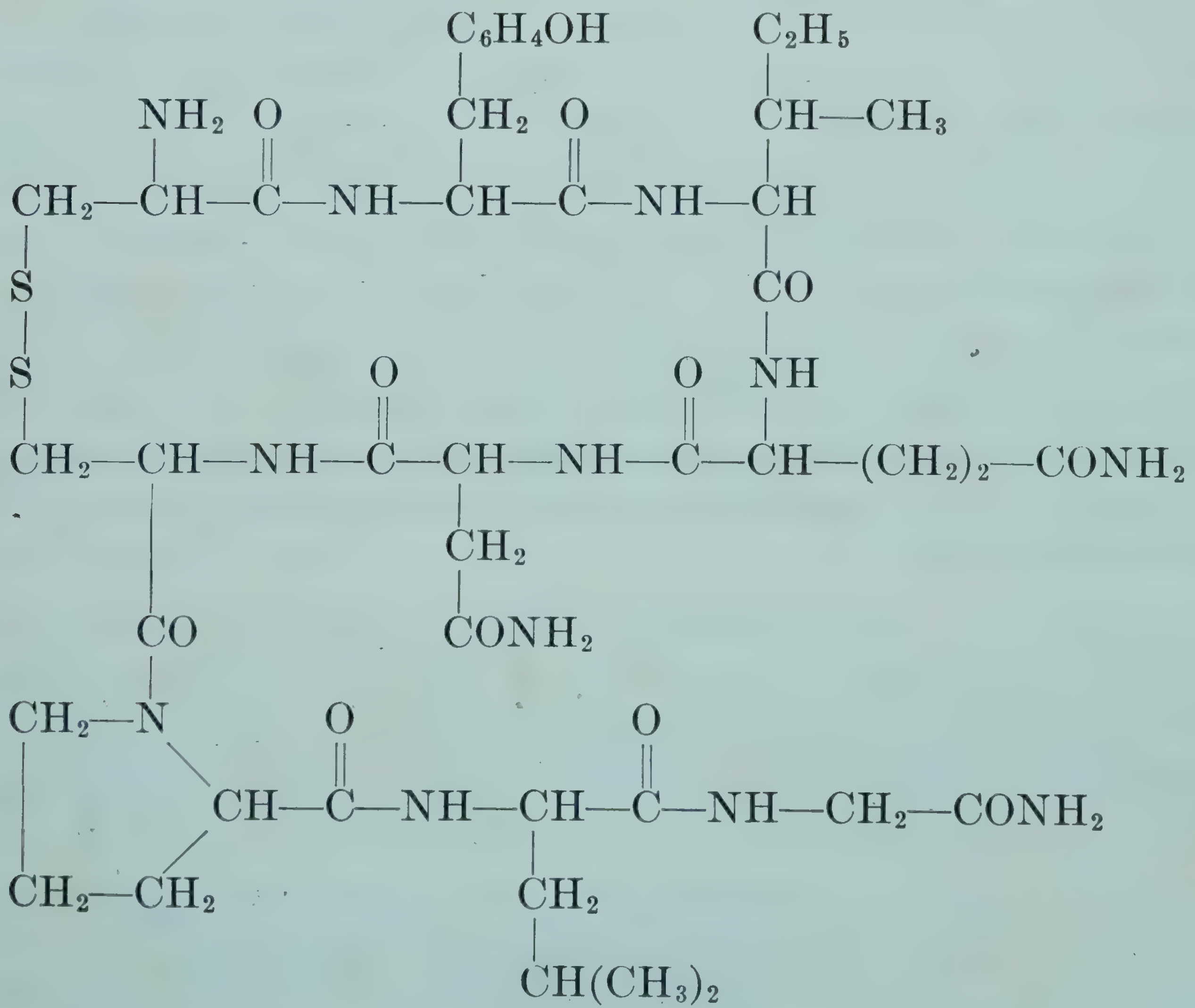
Removal of the whole organ or of the anterior lobe alone produces the following effects (best seen in the rat): cessation of growth; atrophy of the ovaries or testes, which cease to function in producing germ cells and as endocrine organs; atrophy of the thyroid, the reduction of thyroid activity lowering the metabolic rate; atrophy of the adrenal cortex, which may be the cause of an increased susceptibility to toxins, infections, etc., and of a tendency toward dissipation of carbohydrate stores and toward hypoglycemia; and cessation of lactation, if in progress. The administration of suitable extracts, or implantation of anterior-lobe tissue, more or less corrects these deficiencies. It is uncertain how many distinct active principles are present in such extracts, but a number are widely recognized and have been separated in pure or nearly pure state. All are of protein or polypeptide nature, are destroyed by digestive enzymes, and are relatively unstable to heat. There may be interspecific differences in chemical composition rather than in physiological properties, and this may explain the observation that animals treated with anterior-lobe extracts over long periods tend to become unresponsive to them. Clinically, overactivity of the anterior lobe is associated with giantism and acromegaly (often accompanied by symptoms of diabetes and of hyperthyroidism) and congenital underactivity with dwarfism and infantilism. In Simmonds' disease the gland is almost wholly destroyed.

The hormones generally recognized as separate entities are: (1) the *growth* (*somatotropic*) *hormone*, capable of producing accelerated growth in young animals, especially growth in length, and active in metabolism in a "contra-insulin" sense, so as to cause severe hyperglycemia in susceptible animals and sufficient damage to the islets of Langerhans to produce a permanent "metahypophyseal" diabetic state; (2) the *thyrotropic hormone*, stimulating the thyroid gland to activity (reflected in increased height of the epithelial cells, discharge of iodine-containing thyroxine, and hence raised metabolic rate) and producing exophthalmos; (3) the *adrenocorticotropic* hormone (ACTH), stimulating the secretion of glucocorticoids (see p. 758); (4) *prolactin*, the "lactogenic" hormone, which stimulates milk secretion in fully developed mammary glands, and the glandular development of the crop seen in pigeons of both sexes while rearing their young, and which also has the *luteotropic* action of maintaining the structure and endocrine function of the corpora lutea; finally, there are the *gonadotropic hormones*, differentiated as (5) "follicle-stimulating" (FSH) and (6) "luteinizing" or "interstitial-cell-stimulating" (LH or ICSH), which together incite the sex glands to both germinal and endocrine activity (for example, they produce, when injected into immature female rats and mice, premature ripening of follicles, ovulation, formation of corpora lutea, and discharge of ovarian hormones which secondarily affect the uterus and vagina). A substance similar to the luteinizing hormone is produced by the human placenta during pregnancy and is termed *chorionic gonadotropin* (CG) or *prolan;* it is excreted in the urine, and the Aschheim-Zondek, Friedman, and other similar tests, based on this fact, are the most accurate means now available for diagnosing pregnancy in its early stages. These tests also serve for the diagnosis of chorioepithelioma and hydatidiform mole, in which extremely large amounts of gonadotropin are excreted. The serum of pregnant mares (PMS) contains yet another gonadotropin, which though of chorionic origin has a closer resemblance to FSH; it is not excreted in the urine.

Gonadotropic substances (FSH and LH) of pituitary origin may appear in the urine when the sex glands are removed or have ceased to function; so, too, removal or inactivation (e.g., by thiouracil) of the thyroid leads to overproduction of thyrotropic hormone. It is evident that the secretory activity of the anterior lobe is to some extent regulated by the concentration in the blood of the hormones (estrogens, progesterone, androgens, corticoids, thyroxine) produced by its "target organs," so that a self-regulating or "servo" mechanism is set up. These hormones probably influence a center in the brain which controls the anterior lobe by a mechanism partly neural and partly humoral, and which can also be influenced by incoming nervous stimuli (e.g., those arising in the act of mating or the act of suckling) and by higher cerebral centers.

THE POSTERIOR PITUITARY

The posterior lobe contains principles which may be extracted from the acetone-dried gland with dilute acetic acid; *Pituitrin* is a commercial extract of this type. It contains *vasopressin* (*Pitressin*) which, on the one hand, affects the circulation, causing constriction of the capillaries leading to extreme pallor and a tendency to raise blood pressure—offset, however, by an unfavorable action on the heart (coronary constriction)—and, on the other hand, checks the secretion of urine, especially by postponing or abolishing the diuresis normally induced by administration of fluids. *Oxytocin* (*Pitocin*) is also present in Pituitrin but may be almost completely separated from Pitressin; it causes contraction of the smooth muscle of the uterus (except in the presence of an active corpus luteum), and is used therapeutically in the late stages of labor. The extracts also



Oxytocin

affect the intestinal and bronchial musculature, and cause squeezing-out of milk from the ducts of the mammary gland.

The total synthesis of an octapeptide amide having the hormonal activity of oxytocin by du Vigneaud and associates[18] has established a landmark since it is the first polypeptide hormone to have been produced in the laboratory. Its molecular weight is approximately 1000; the molecule consists of eight amino acid equivalents (leucine, isoleucine, tyrosine, proline, glutamic acid, aspartic acid, glycine, and cystine) and three equivalents of ammonia, the structure being in part cyclic. (See p. 775.)

The synthesis of the vasopressor-antidiuretic hormone of the posterior pituitary by the Cornell investigators appears imminent.[19]

If the posterior lobe is extirpated, or if the nerve fibers which run into it from the nucleus supraopticus are severed, there is a permanent increase in urine flow and in water consumption (diabetes insipidus). The posterior lobe principles arise in the pars nervosa and are polypeptides; they are unstable in alkaline solution and inactive by mouth. There is evidence that, as ordinarily obtained, they may be breakdown products of a much larger molecule possessing both pressor and oxytocic activity. The pars intermedia, which is embryologically part of the anterior lobe, produces a substance (*intermedin*) which causes expansion of pigment masses in certain cells (melanophores, erythrophores) in the skin of lower vertebrates. A frog from which the pituitary is removed is rendered permanently pale in color by contraction of the melanophores. It is uncertain whether this substance has any function in mammals.

EXPERIMENTS ON THE PITUITARY

1. *Preparation of Anterior Lobe Extract: Method of Evans.*[20] Bovine anterior lobes, as fresh as possible, are ground in a mortar with twice their weight of sand; 2 ml. of water are added for every g. of gland. The volume of the mixture is measured and three-eighths of this quantity of 0.2 N NaOH is added; the material is kept for 12 hours in the icebox; the supernatant fluid is decanted off and neutralized to phenol-red with 0.2 N acetic acid. Crude extracts of this type contain maximal amounts of the several hormones, except that positive stimulation of the sex glands is not very readily obtainable with bovine material. It is, however, still easier to dehydrate dissected anterior lobes in the lyophilizing apparatus; when dry the tissue crumbles to a light, relatively stable powder, from which uniform suspensions are easily prepared. It may be advantageous to add small amounts of penicillin and streptomycin, so as to lessen the risk of abscesses forming at the site of injection of the suspension.

2. *Preparation of Chorionic Gonadotropin: Method of Katzman and Doisy.*[21] Human pregnancy urine (preserved with a little chloroform and kept at 1 to 5° C.) is made acid to methyl red–methylene blue (pH 4–5) with glacial acetic acid and filtered; 50 ml. of a saturated solution of benzoic acid in acetone are added to each liter of urine, with vigorous stirring, and the mixture

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[18] du Vigneaud, Ressler, Swan, Roberts, Katsoyannis, and Gordon: *J. Am. Chem. Soc.*, 75, 4879 (1953).

[19] du Vigneaud, Lawler, and Popenoe: *J. Am. Chem. Soc.*, 75, 4880 (1953).

[20] Evans and Simpson: *Am. J. Physiol.*, 98, 511 (1931).

[21] Katzman and Doisy: *J. Biol. Chem.*, 98, 739 (1931).

is allowed to stand overnight. The precipitated benzoic acid, upon which the active material is adsorbed, is filtered off with suction and dissolved in a volume of acetone equal to the amount originally added. A small flocculent precipitate of acetone-insoluble material contains the active principle and is separated by decantation and centrifuging and thoroughly washed with acetone. Small additional quantities may be obtained by repeating the benzoic acid adsorption on the urine filtrate. The active principle is extracted from the combined precipitates by three treatments with distilled water, centrifuging after each extraction, and using not more than 25 ml. of water per liter of urine. The water-insoluble residue is discarded. Other useful methods involve adsorption upon Lloyd's reagent[22] or precipitation with tungstic acid.[23] All these methods may be used to concentrate the activity in urines other than those of pregnancy, when the gonadotropic substance is too dilute to be determined by direct biological assay of the urine.

3. *Detection of Chorionic Gonadotropin (Prolan): Pregnancy Test of Aschheim and Zondek.*[24] Five baby female mice, 6 to 8 g. in weight and 3 to 4 weeks old, are injected with the urine to be tested. This should be taken from the first sample passed in the morning, and toxic substances may be removed from it by shaking with ether. Each mouse is given 6 injections of 0.5 ml. of this urine, 3 doses on the first day and 3 on the second, and the animals are killed 96 hours after the beginning of the test. The reaction sought consists of three parts: (I) Formation of large ovarian follicles and precocious appearance of estrus; (II) hemorrhagic follicles, easily seen under a lens as deep clear red spots; (III) formation of corpora lutea, visible under a lens. Reaction I by itself is insufficient to establish a diagnosis of pregnancy. The dependability of the reaction is at least 98 per cent. It usually becomes positive a few days after the first missed menstrual period; in the ensuing month, the concentration of the active principle rises to a well-marked maximum. Very high concentrations occur in the presence of tumors of placental tissue (hydatid mole or chorionepithelioma).

4. *Friedman Pregnancy Test.*[25] This test is more rapidly completed, is equally accurate, and probably more convenient where tests are made only occasionally. It is based on the fact that rabbits ovulate only when their ovaries are specifically stimulated, as they are after mating. A mature female rabbit, which must have been isolated in a cage by itself for at least three weeks previously, is given 10 ml. of urine to be tested by injection into the marginal ear vein. The ovaries are examined 24 hours later; a positive reaction is marked by reddish protrusions or by recent hemorrhage in the follicles. The abdomen may be opened and the ovaries inspected under anesthesia, so that the rabbit may be used again some weeks later, when the corpora lutea formed have disappeared.

THE KIDNEY

When the kidneys or the arteries supplying them are compressed, there follows a sustained rise in systemic blood pressure, resembling the clinical entity of essential hypertension; the phenomenon is independent of nervous connections and of changes in excretory function. It is ascribed to the secretion into the blood of a globulin *renin*, which appears to be a proteolytic enzyme and to vary in composition from species to species. The substrate for renin is a fraction (*hypertensinogen* or "activator") of the serum globulins, and the product of the reaction a heat-stable, dialyzable vasoconstrictor, probably a polypeptide, called *hypertensin* or *angiotonin*; this in turn is slowly destroyed by a substance ("inhibitor," *angiotonase*, or *hypertensinase*) present in normal blood and tissues.

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[22] Davy and Sevringhaus: *Proc. Soc. Exptl. Biol. Med.*, **30**, 1422 (1933).

[23] Katzman and Doisy: *Proc. Soc. Exptl. Biol. Med.*, **31**, 188 (1938).

[24] Aschheim: *J. Am. Med. Assoc.*, **104**, 1324 (1935).

[25] Friedman and Lapham: *Am. J. Obstet. Gynecol.*, **21**, 405 (1931).

THE THYMUS AND PINEAL

Endocrine function has frequently been ascribed to these organs, but has not been established; extirpation, even in very young animals, has no specific systemic effects.

OTHER HORMONES

The first hormone whose function was clearly established (by Bayliss and Starling in 1903) was *secretin*. This is a substance which is liberated from the walls of the duodenum when acid chyme enters the lumen; it is carried by the blood to the pancreas, where it stimulates the secretion of pancreatic juice. Tests on the most highly purified secretin show it to be a polypeptide. See also p. 393.

Less well established are *cholecystokinin*, believed to arise in the same source and to stimulate contraction of the gallbladder; *enterogastrone*, believed to inhibit the secretory and motor activity of the stomach when fat is present in the intestine; and *gastrin*, which physiologically resembles histamine, and may evoke secretion of hydrochloric acid by the fundus of the stomach when the pyloric region is stimulated by the presence of partially digested food.

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27

Urine: General Characteristics of Normal and Pathological Urine

Secretion of Urine. The problem of the mechanism of the formation of urine by the kidney has occupied the attention of investigators for many years, and has produced many conflicting theories. The filtration-reabsorption theory of Cushny has been the basis of the modern theory, and the work of Starling, Marshall, Richards, and their collaborators and of other investigators has led to a more satisfactory conception of the processes involved.

Marshall has summarized the known facts relating to the formation of urine by the mammalian kidney in the following working hypothesis: "All of the non-colloid constituents of the plasma are eliminated by filtration through the glomerulus; water, chloride, bicarbonate, potassium (possibly phosphate, uric acid, and other bodies) are reabsorbed during the passage of the filtrate along the tubules; urea and sulfates are also reabsorbed to some extent under certain conditions but not as actively as chloride; ammonia, hippuric acid, and possibly other bodies are formed in the renal cells and secreted, while certain foreign substances when present in the organism (phenol red) and possibly substances occurring in small amounts in the organism are secreted after a preliminary concentration in the tubule cells. The amount of glomerular filtrate on this hypothesis is considered sufficient to account for all of the sulfate and urea eliminated but not sufficient to account for ammonia, certain dyes (phenol red, etc.) and other bodies which are concentrated to a much greater extent than sulfate by the kidney."

By direct catheterization of the glomerular urine of the frog and comparison of its composition with simultaneously collected bladder urine, Wearn and Richards formulated a hypothesis illustrated diagrammatically in Fig. 213. It will be noted that the chief difference from Marshall's conception is in the site of dye secretion, these workers having found small amounts of dye in the glomerular filtrate although they admit much of the neutral red excretion is carried out by the tubules. They also found the polysaccharide inulin to be excreted by the glomerulus, indicating the presence of pores of considerable size in the membrane.

Reabsorption of water, which may be as high as 97 per cent, appears to occur in both the proximal and distal tubules. Chloride is absorbed preferentially in the distal tubule, where also takes place the reabsorption of bicarbonate and acidification of the urine, probably through excretion of hydrogen ions.[1] Reabsorption of glucose occurs in the proximal tubule, chiefly through esterification with phosphate. The substance phlorizin prevents this esterification and hence permits glucose to pass into the urine ("phlorizin diabetes"). Nitrogenous waste products appear in the urine either through failure of reabsorption from the glomerular filtrate or through actual secretion by the tubular cells or both. Ammonia appears definitely to be secreted by the tubular cells, some urea may arise in this manner, and there is evidence for the tubular secretion of creatinine by man and Necturus but not by the frog. Thus species differences must be considered in interpreting kidney action.

Volume. The volume of urine excreted by normal individuals during any definite period fluctuates within very wide limits. The total volume of glomerular filtrate produced by normal adult kidneys may be 75 to 150 liters in 24 hours. However, after tubular reabsorption the average normal excretion of urine falls within the range of 1000 to 2000 ml. The volume excretion is influenced greatly by the diet, particularly by the ingestion of fluids, and by the ambient temperature, which affects not only fluid intake but also loss of water through perspiration. Strenuous physical exercise causes a diminished output of urine.

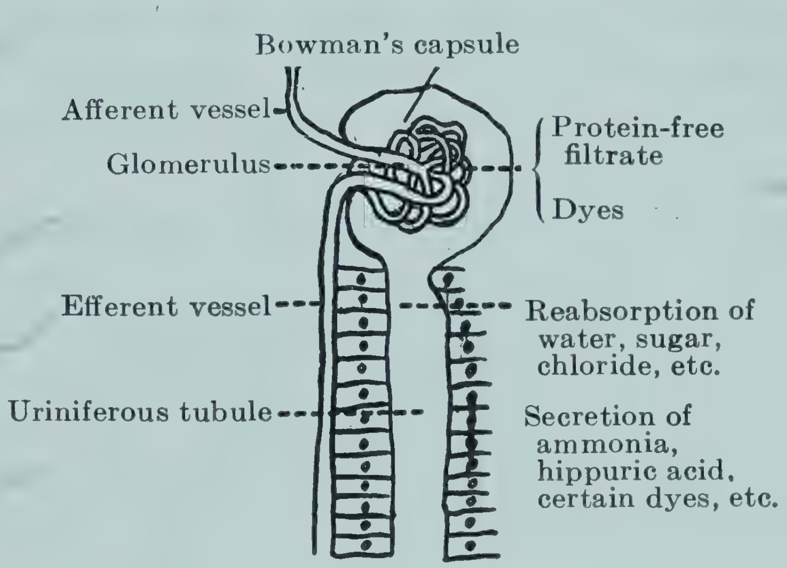
Bowman's capsule
Afferent vessel
Glomerulus
Protein-free filtrate
Dyes
Efferent vessel
Reabsorption of water, sugar, chloride, etc.
Uriniferous tubule
Secretion of ammonia, hippuric acid, certain dyes, etc.

FIG. 213. DIAGRAM ILLUSTRATING THE FORMATION OF URINE.

Certain pathological conditions cause the output of urine for any definite period to depart very decidedly from the normal output. If the output is increased the condition is termed *polyuria*, whereas a diminished excretion is termed *oliguria*. Among the pathological conditions in which the volume of urine is *increased* above normal are the following: diabetes mellitus, diabetes insipidus, certain diseases of the nervous system, contracted kidney, amyloid degeneration of the kidney, and in convalescence from acute diseases in general. Many drugs such as calomel, digitalis, acetates, and salicylates also increase the volume of the urine excreted. A *decrease* from the normal is observed in the following pathological conditions: acute nephritis, diseases of the heart and lungs, fevers, diarrhea, and vomiting.

Color. Normal urine ordinarily possesses an amber yellow tint, the depth of the color being dependent in part upon the density of the fluid.

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[1] Pitts and Alexander: *Am. J. Physiol.*, **144**, 239 (1945).

The color of normal urine is due principally to a pigment called urochrome. Traces are generally present of *uroerythrin*, a red pigment possibly derived from the melanins, *coproporphyrins*, and *uroporphyrin*, iron-free reddish pigments resulting from the metabolism of heme. In *porphyria*, a disorder of porphyrin metabolism, the porphyrin content is increased. In certain other pathological urines a chromogen (indoleacetic acid) may be present. This yields urorosein, a reddish pigment, when the urine is strongly acidified (see p. 852). Urochrome is said to be a compound of urobilin and urobilinogen with a peptide substance. The amount excreted per day by an adult man has been estimated at 7.3 mg., being very constant for a given individual. Under pathological conditions or after the administration of various drugs or antiseptics, the color of the urine may vary in intensity from an extremely light yellow to a very dark brown or black or may even assume the color of the drugs or their degradation products. Vogel has constructed a color chart which is of some value for purposes of comparison. The nature and origin of the chief variations in the urinary color are set forth in tabular form on p. 783.

Turbid Urine. Normal urine is ordinarily perfectly clear and transparent when voided. On standing for a variable time, however, a cloud (nubecula) consisting principally of nucleoprotein or mucoid (see p. 810) and epithelial cells forms. A turbidity due to the precipitation of phosphates is normally noted in urine passed after a hearty meal. The urine obtained two to three hours after a meal or later is ordinarily free from turbidity. Permanently turbid urines ordinarily arise from pathological conditions.

Odor. The odor of normal urine is of a faint, aromatic type. The substances to which this odor is due are not well known, but it is claimed by some investigators to be due, at least in part, to the presence of minute amounts of certain volatile organic acids. When the urine undergoes decomposition, e.g., in alkaline fermentation, a very unpleasant ammoniacal odor is evolved. All urines are subject to such decomposition if allowed to stand for a sufficiently long time. Under normal conditions the urine very often possesses a peculiar odor due to the ingestion of some certain drug or vegetable. For instance, cubebs, copaiba, myrtol, saffron, tolu, and turpentine each impart a somewhat specific odor to the urine. After the ingestion of asparagus, the urine also possesses a typical odor attributed to methyl mercaptan ($CH_3 \cdot SH$) which may, however, exist in urine only as a precursor which yields the mercaptan on heating in acid solution.

Frequency of Urination. The frequency of urination varies greatly in different individuals, but in general is dependent upon the amount of fluid in the bladder. In pathological conditions an inflammatory affection of the urinary tract or any disturbance of the innervation of the bladder will influence the frequency. Affections of the spinal cord which lead to an increased irritability of the bladder or a weakening of the sphincter, or any condition lowering the residual capacity of the bladder, will result in increasing the frequency of urination. It often aids in diagnosis.

Reaction. The mixed 24-hour urinary excretion of a normal individual ordinarily possesses an acid reaction to litmus. The actual hydrogen-ion

Color	*Cause of Coloration*	*Pathological Condition*
Nearly colorless.	Dilution, or diminution of normal pigments	Nervous conditions: hydruria, diabetes insipidus, granular kidney
Dark yellow to brown red. .	Increase of normal, or occurrence of pathological, pigments. Concentrated urine	Acute febrile diseases
Milky.	Fat globules	Chyluria
	Pus corpuscles	Purulent diseases of the urinary tract
Orange.	Excreted drugs	Santonin, chrysophanic acid
Red or reddish.	Uroerythrin, uroporphyrin, coproporphyrin; hemoglobin and myoglobin	Porphyrin, hemorrhages, hemoglobinuria, trauma
	Pigments in food (logwood, madder, bilberries, fuchsin)	
Brown to brown black.	Hematin	Small hemorrhages
	Methemoglobin	Methemoglobinuria
	Melanin	Melanotic sarcoma
	Hydroquinol and catechol	Phenol poisoning
Greenish yellow, greenish brown, approaching black	Bile pigments	Jaundice
Dirty green* or blue.	A dark blue scum on the surface, with a blue deposit, due to an excess of indigo-forming substances	Cholera, typhus; seen especially when the urine is putrefying
Brown yellow to red brown, becoming blood red upon adding alkalis.	Substances contained in senna, rhubarb, and chelidonium which are introduced into the system	

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* This dirty green or blue color also occurs after the use of methylene blue in the organism.

concentration varies over a wide range (pH 4.8 to 8.0), the mean being about pH 6. The reaction of the urine represents an equilibrium among a large number of acid and basic constituents, both organic and inorganic, which it contains. Although organic acids and bases play a part in producing the normal reaction, this reaction is probably, in the main, dependent upon the relative amounts of the mono- and dibasic sodium and potassium phosphates present. The monobasic sodium phosphate (NaH_2PO_4) is acid in reaction, while the dibasic phosphate (Na_2HPO_4) is alkaline in reaction. The excretion of acid or alkaline phosphate by the kidneys is one of the factors in the regulation of the neutrality of the blood and of the organism in general. The acidity of the urine, as determined by titration, runs in general parallel with the hydrogen-ion concentration and seems to be dependent upon the same factors, and in more acid urines mainly on the phosphate content. Van Slyke and Palmer have shown that normal men excrete organic acids equivalent to only about 6 ml. of 0.1 N acid per kilo in 24 hours. Strenuous physical exercise produces an increase in hydrogen-ion concentration and in acid and ammonia output. (For further discussion of acidity, see Chapter 31.)

The mean acidity in cardiorenal diseases is high—about pH 5.3 as compared with pH 6, the normal mean. In general the acidity tends to be increased in the greater number of pathological disorders.

The composition of the food is perhaps the most important factor in determining the reaction of the urine (see Chapter 34, "Inorganic Metabolism," for the influence of base-forming and acid-forming foods). The reaction ordinarily varies considerably according to the time of day the urine is passed. For instance, for a variable length of time after a meal the urine may be neutral or even alkaline in reaction to litmus, owing to the claim of the gastric juice upon the acidic radicals to further the formation of hydrochloric acid for use in carrying out the digestive secretory function. This hypothesis has been verified experimentally. This change in reaction is known as the *alkaline tide* and is common to perfectly healthy individuals. The urine may also become temporarily alkaline in reaction as the result of ingesting alkaline carbonates or certain salts of tartaric and citric acids which ultimately yield bicarbonate within the organism. Ingestion of acid fruits (oranges, lemons, peaches, etc.) causes the formation of alkaline urine. This is due to the fact that the ash of such fruits is alkaline and when the fruits are combusted in the body bicarbonate is formed. On the other hand, bread, cereals, meats, etc., yield an acid ash and an acid urine. Certain acid fruits, like cranberries, plums, and prunes, increase urinary acidity. This is due to the fact that these fruits contain considerable quantities of *quinic acid*. Instead of being oxidized, this is converted into hippuric acid, which renders the urine acid in reaction.

Normal urine upon standing for some time becomes alkaline owing to the inception of alkaline or ammoniacal fermentation through the agency of microorganisms. This fermentation has no especial diagnostic value except in cases where the urine has undergone this change *within the organism* and is voided in the decomposed state. Ammoniacal fermentation is ordinarily due to cystitis or occurs as the result of infection in the process of catheterization. A microscopical examination of such urine (Fig. 214) shows the presence of ammonium magnesium phosphate crystals, amorphous phosphates, and not infrequently ammonium urate.

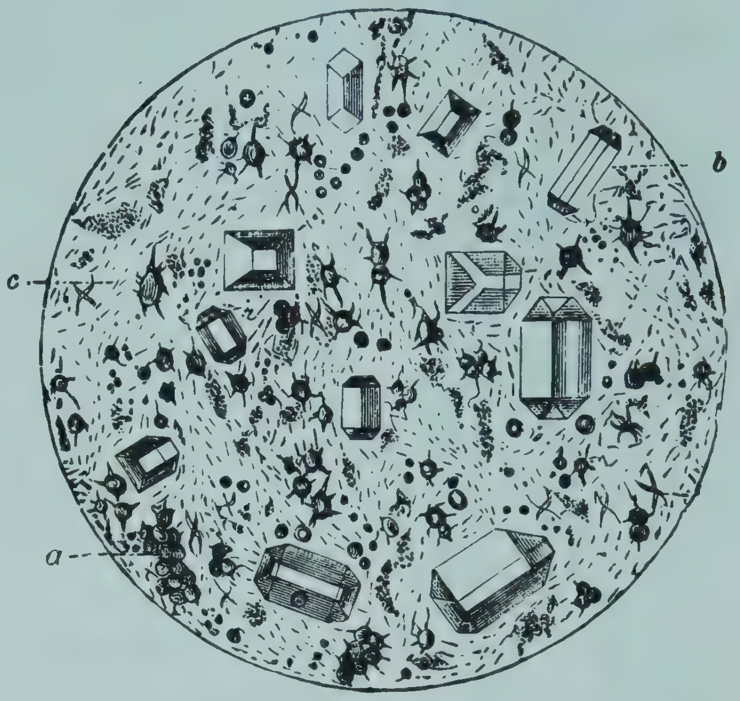

FIG. 214. DEPOSIT IN AMMONIACAL FERMENTATION.

a, Acid ammonium urate; *b*, ammonium magnesium phosphate; *c*, bacteria.

Occasionally a urine which possesses a normal acidity when voided will upon standing, instead of undergoing ammoniacal fermentation as above described, become more strongly acid in reaction. Such a phenomenon is termed *acid fermentation.* Accompanying this increased acidity there is ordinarily a deepening of the tint of the urinary color. Such urines may contain acid urates, uric acid, fungi, and calcium oxalate (Fig. 215). On standing for a sufficiently long time any urine which exhibits acid fermentation will ultimately change in reaction owing to the inception of alkaline fermentation, and will show the microscopic deposits characteristic of such a urine.

Specific Gravity. The specific gravity of the urine of normal individuals varies ordinarily between 1.015 and 1.025. This value is subject to wide fluctuations under various conditions. For instance, following copious water- or beer-drinking the specific gravity may fall to 1.003 or lower, whereas in cases of excessive perspiration it may rise as high as 1.040 or even higher. Where a very accurate determination of the specific gravity is desired, use is commonly made of the pycnometer or of the Westphal hydrostatic balance. These instruments, however, are not

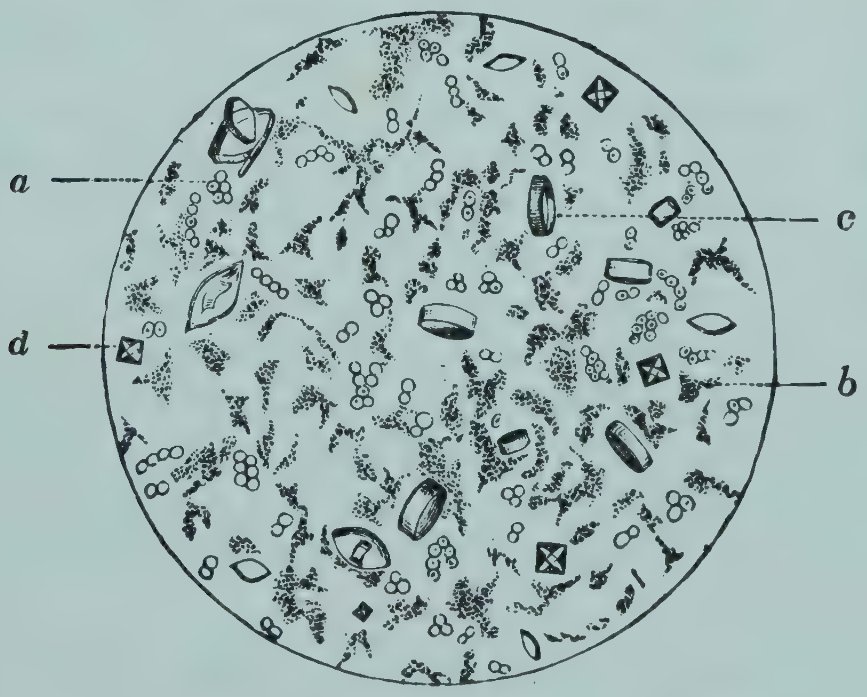

FIG. 215. DEPOSIT IN ACID FERMENTATION.

a, Fungus; *b*, amorphous sodium urate; *c*, uric acid; *d*, calcium oxalate.

suited for clinical use. The clinical method of determining the specific gravity is by means of a urinometer (Fig. 216). This affords a very rapid method and at the same time is sufficiently accurate for clinical purposes. The urinometer is always calibrated for use at a specific temperature and the observations made at any other temperature must be subjected to a certain correction to obtain the true specific gravity. In making this correction one unit in the third decimal place is added to the observed specific gravity for every three degrees centigrade above the normal temperature and subtracted for every three degrees below the normal temperature. For instance, if in using a urinometer calibrated for 15° C. the specific gravity of a urine having a temperature of 21° C. is determined as 1.018, it is necessary to add to the observed specific gravity 2 × 0.001 to obtain the real specific gravity of the urine. Therefore the specific gravity at 15° C. of a urine having a specific gravity of 1.018 at 21° C., would be 1.018 + 0.002 = 1.020.

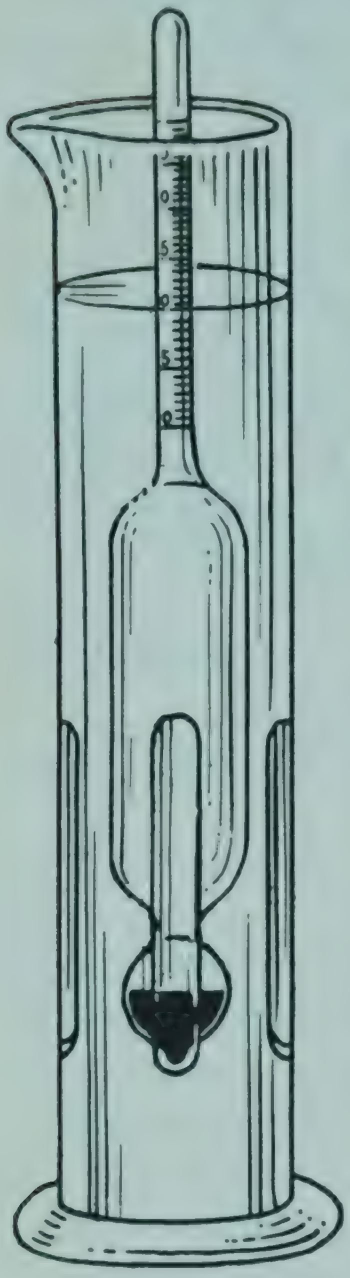

FIG. 216. URINOMETER AND CYLINDER.

Pathologically, the specific gravity may be subjected to very wide variations. This is especially true in diseases of the kidneys. In acute nephritis ordinarily the urine is concentrated and of a high specific gravity, whereas in chronic nephritis the reverse conditions are more apt to prevail. In fact, under most conditions, whether physiological or pathological, the specific gravity of the urine is inversely proportional to the volume excreted. This is not true of diabetes mellitus, however, in which the volume of urine is large and the specific gravity also is high, owing to the sugar contained in the urine.

The total solids normally excreted in the urine may be roughly calculated by means of Long's coefficient, i.e., 2.6. The solids content of 1000 ml. of urine is obtained by multiplying the last two figures of the specific gravity observed at 25° C. by 2.6. To determine the amount of solids excreted in 24 hours if the volume was 1120 ml. and the specific gravity was 1.018, the calculation would be as follows:

(*a*) $18 \times 2.6 = 46.8$ g. of solid matter in 1000 ml. of urine

(*b*) $\dfrac{46.8 \times 1120}{1000} = 52.4$ g. of solid matter in 1120 ml. of urine

Collection and Preservation of the Urine Sample. If any dependable data are desired regarding the quantitative composition of the urine, the examination of the mixed excretion for 24 hours is absolutely necessary. In collecting the urine the bladder may be emptied at a given hour, say 8 A.M., the urine discarded, and all the urine from that hour up to and including that passed the next day at 8 A.M., saved, thoroughly mixed, and a sample taken for analysis.

Toluene is a very satisfactory preservative for urine. In using this preservative simply overlay the urine with a thin layer of the toluene. Formaldehyde (2 drops per 50 ml. of urine) or a bit of camphor or thymol are also satisfactory urine preservatives which do not interfere with the tests for the major urinary constituents.

Another satisfactory preservative consists of a 3:2 mixture of hexamethylenetetramine (urotropin) and salicylic acid, which is used in the proportion of 50 mg. per 10 ml. of urine. These substances produce formaldehyde in solution and, it is claimed, do not interfere with any of the usual tests.

In certain pathological conditions it is desirable to collect the urine passed during the day separately from that passed during the night. When this is done, the urine voided between 8 A.M. and 8 P.M. may be taken as the day sample and that voided between 8 P.M. and 8 A.M. as the night sample.

The qualitative testing of urine samples collected at random, except in a few specific instances, is of no particular value so far as giving us any accurate knowledge as to the exact urinary characteristics of the individual is concerned. In the great majority of cases the qualitative as well as the quantitative tests should be made upon the mixed excretion for a 24-hour period as well as upon a night sample as above described.

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28

Urine: Physiological Constituents[1]

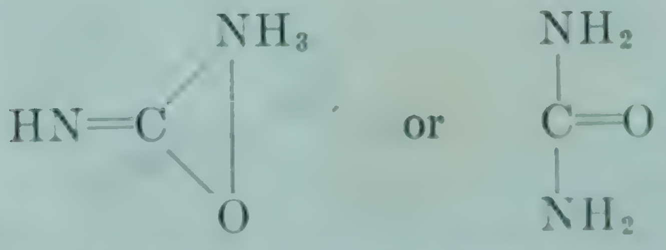
Normal urine varies widely in composition, being influenced by diet and other factors. The following table represents the composition of average normal urine.

COMPOSITION OF A TYPICAL NORMAL URINE

Constituent	*Daily Excretion in Grams*
Water	1200.0
Solids	60.0
Urea	30.0
Uric acid	0.7
Hippuric acid	0.7
Creatinine	1.2
Indican	0.01
Oxalic acid	0.02
Allantoin	0.04
Amino acid nitrogen	0.2
Purine bases	0.01
Phenols	0.2
Chloride as NaCl	12.0
Sodium	4.0
Potassium	2.0
Calcium	0.2
Magnesium	0.15
Sulfur, total, as S	1.0
Inorganic sulfates as S	0.8
Neutral sulfur as S	0.12
Conjugated sulfates as S	0.08
Phosphate as P	1.1
Ammonia	0.7

ORGANIC PHYSIOLOGICAL CONSTITUENTS

UREA



$HN{=}C(NH_3)(O)$ or $NH_2{-}C({=}O){-}NH_2$

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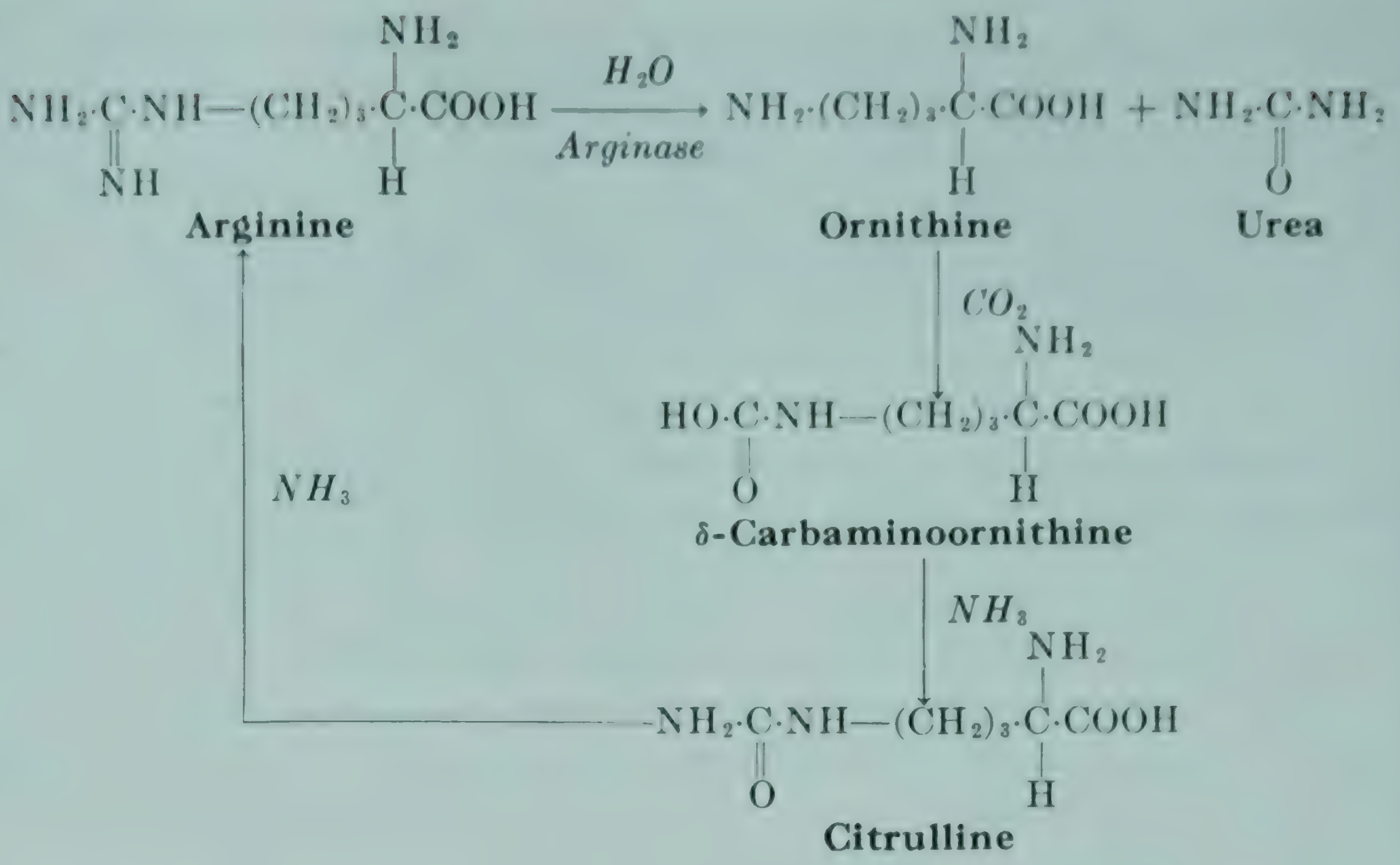
[1] It is impossible to make an absolute distinction between physiological and pathological constituents of the urine. A substance may be present in urine in small amount physiologically and be sufficiently increased under certain conditions to be termed a pathological constituent. Therefore it depends, in some instances, upon the quantity of a constituent present whether it may be correctly termed a physiological or a pathological constituent.

Urea is the principal end product of the metabolism of protein substances in mammals, amphibia, and elasmobranch fishes. About 80 to 90 per cent of the total nitrogen of human urine is present as urea. The distribution of the nitrogen of the urine among urea and the other nitrogen-containing compounds present depends upon the absolute amount of the total nitrogen excreted. A decrease in the total nitrogen excretion is always accompanied by a decrease in the percentage of the total nitrogen excreted as urea. By so reducing the protein content of the diet of a normal person as to cause the excretion of total nitrogen to be reduced to 3 to 4 g. in 24 hours, *only about 60 per cent of this nitrogen appears in the urine as urea.* Urea is the only one of the nitrogenous excretions which is decreased, relatively as well as absolutely, as a result of decreasing the amount of protein metabolized. Folin reported a hospital case in which only 14.7 per cent of the total nitrogen was present as urea and about 40 per cent was present as ammonia. Mörner had previously reported a case in which but 4.4 per cent of the total nitrogen of the urine was present as urea, and 26.7 per cent was present as ammonia.

Urea occurs most abundantly in the urine of man and carnivora and in somewhat smaller amount in the urine of herbivora; the urine of fishes, amphibians, and certain birds also contains a small amount of the substance. Urea is also found in nearly all the fluids and in many of the tissues and organs of mammals. The amount excreted under normal conditions by an adult man in 24 hours is about 30 g. The excretion is greatest in amount on a diet of meat, and least in amount on a diet consisting of nonnitrogenous foods; this is due to the fact that the urea output is regulated by the protein metabolism. A low-protein diet has a tendency to decrease the metabolism of the tissue proteins and thus the output of urea under these conditions may fall below that observed during starvation. The output of urea is also increased after copious water- or beer-drinking. The increase is probably due primarily to the washing out of the tissues of the urea previously formed, but which had not been removed in the normal processes, and secondarily to a stimulation of protein catabolism.

The formation of urea appears to be a property of the liver exclusively. This has been demonstrated by experiments in which there was complete cessation of urea formation after extirpation of the liver. Studies with isolated tissues, incubated in thin slices under physiological conditions, have also shown that only liver tissue is capable of synthesizing urea.

The mechanism of urea formation by the liver is still uncertain. Early views included formation from free NH_3, presumably liberated from amino acids by oxidative deamination, through the intermediate formation of ammonium carbonate, $(NH_4)_2CO_3$, or ammonium carbamate, $NH_4 \cdot O \cdot CO \cdot NH_2$. Attention has shifted to the role of certain amino acids in urea formation because of the studies of Krebs and Henseleit. These investigators, working with liver slices, showed that the liver could synthesize urea from ammonia and postulated the existence of a cyclic mechanism including ammonia, carbon dioxide, and the amino acids arginine, ornithine, and citrulline. The steps in the process, according to the *ornithine cycle* theory, are as follows:



Thus the primary source of urea according to this theory is the action of the enzyme *arginase* on the amino acid arginine, to produce urea and ornithine. The ornithine acts as a catalyst, being converted back to arginine by the reactions shown, taking up ammonia and carbon dioxide in the process which ultimately appear as urea. This theory, while attractive, is not universally accepted (see Chapter 33). Points in its favor include the well-known and powerful arginase activity of liver, and the fact that citrulline, first isolated from watermelon juice, is found in small amount in the blood, and its synthesis by liver tissue can be demonstrated. Opposed to it is the fact that the experimental demonstration of the ornithine cycle with liver slices requires the presence of free ammonia in far higher concentration than is ever encountered in living tissue; even moderate amounts of free ammonia are not acted on at all by liver slices. Thus, if the theory is correct, the nitrogen would appear to enter the cycle in some other way than as free ammonia. Further evidence on the subject is clearly required.

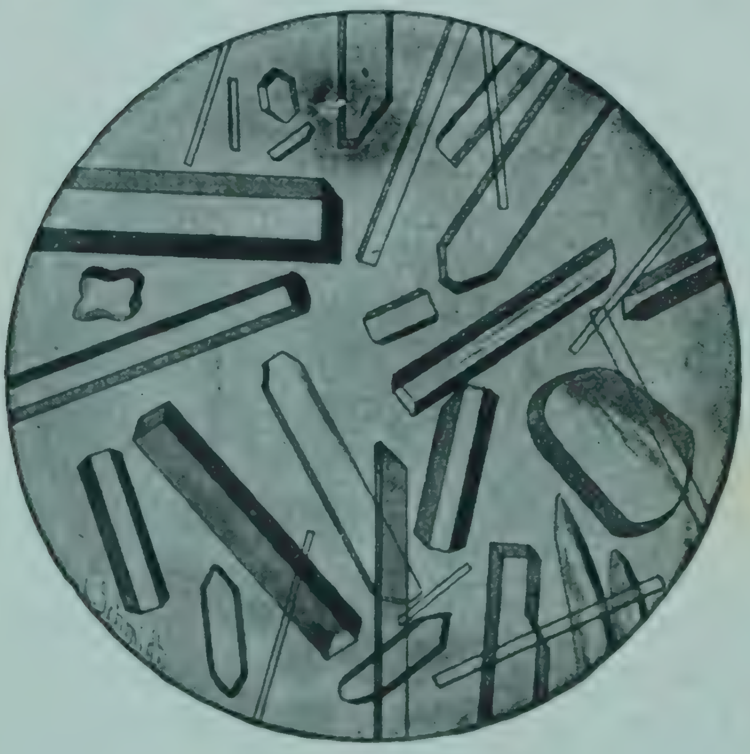
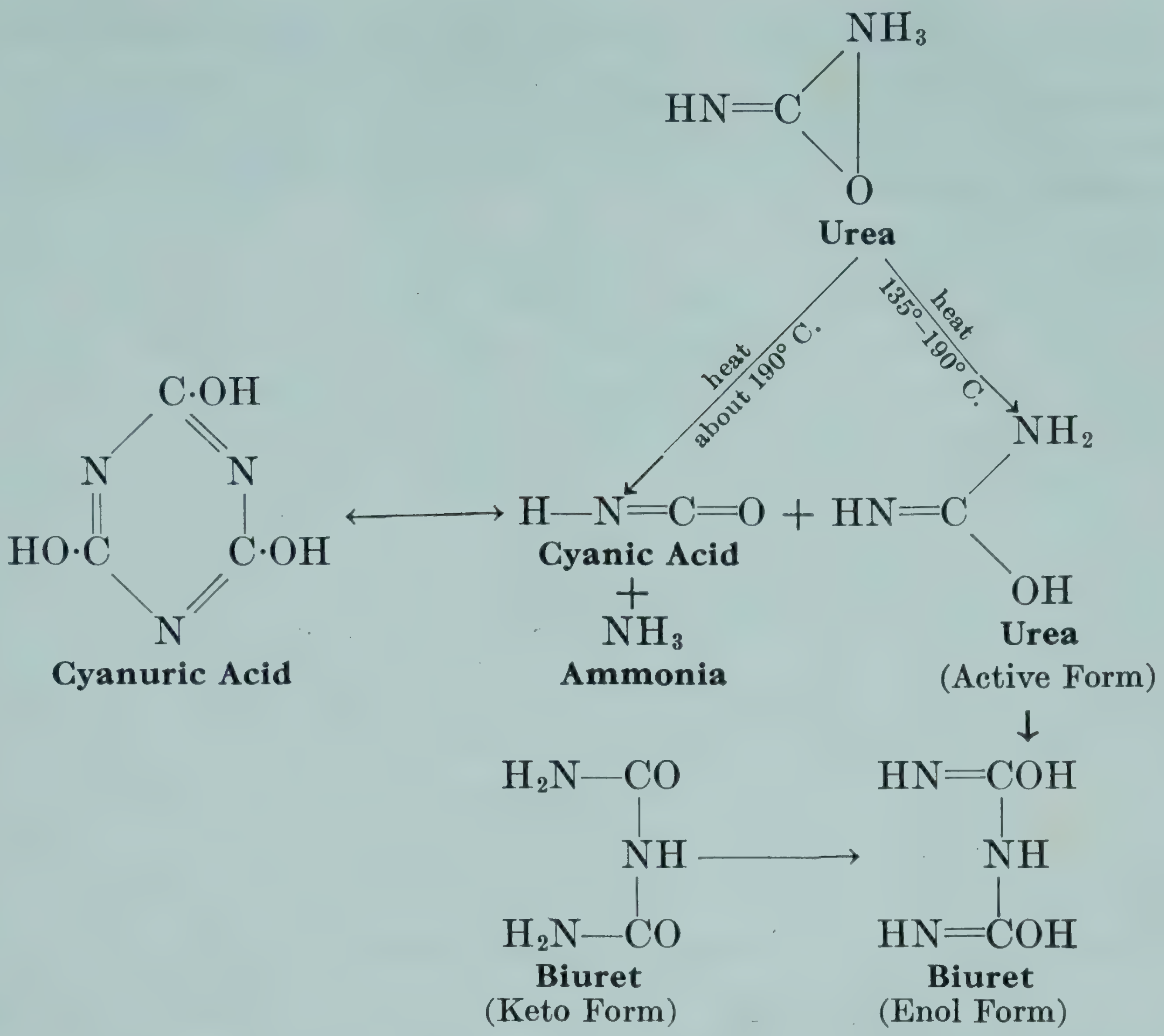


FIG. 217. UREA.

Urea crystallizes in long, colorless, four- or six-sided, anhydrous, rhombic prisms (Fig. 217), which melt at 132° C. and are soluble in water or alcohol and insoluble in ether or chloroform. If urea is heated in a test tube, it melts and decomposes with the liberation of ammonia and the formation of biuret and cyanuric acid. According to the Werner hypothesis the changes are as follows:



The biuret may be dissolved in water and a reddish-violet color obtained by treating the aqueous solution with copper sulfate and potassium hydroxide (see "Biuret Test," p. 171). Certain hypochlorites or hypobromites in alkaline solution decompose urea into nitrogen, carbon dioxide, and water. Sodium hypobromite brings about this decomposition as follows:

$$CO(NH_2)_2 + 3NaOBr \rightarrow NaBr + N_2 + CO_2 + 2H_2O$$

According to the Werner hypothesis, the urea is momentarily brominated and the resultant compound hydrolyzed by the alkali and oxidized by hypobromite. Sodium cyanate, sodium nitrate, hydrazine, and CO are formed as by-products, thus accounting for the low nitrogen values obtained in the clinical application of this method.

Soybeans, jack beans, and watermelon seeds contain an enzyme called urease which has the power to decompose urea with the liberation of ammonia. This fact is made use of in the quantitative determination of urea (see Chapter 23). Urease action appears to involve the intermediate formation of ammonium carbamate and not of cyanate.

Urea forms crystalline compounds with certain acids; urea nitrate and urea oxalate are the most important. Urea nitrate, $CO(NH_2)_2{\cdot}HNO_3$, crystallizes in colorless rhombic or six-sided tiles (Fig. 218), which are easily soluble in water. Urea oxalate, $(CO(NH_2)_2)_2{\cdot}H_2C_2O_4$, crystallizes in the form of rhombic or six-sided prisms or plates (Fig. 219): the oxalate differs from the nitrate in being somewhat less soluble in water.

A decrease in the excretion of urea is observed in many diseases in which the diet is much reduced, in diseases associated with impaired liver function, in some disorders as a result of alterations in metabolism, e.g., myxedema, but most frequently as a result of diminished excretion, as in severe and advanced kidney disease. In fact, the determination of the ability of the kidneys to excrete urea (the "urea clearance" test, see Chapter 31) is perhaps the most valuable single clinical index of renal function. A pathological increase may result from tissue catabolism in febrile or wasting conditions. In marked acidosis it may be considerably decreased relative to the total nitrogen (see "Ammonia," p. 814).

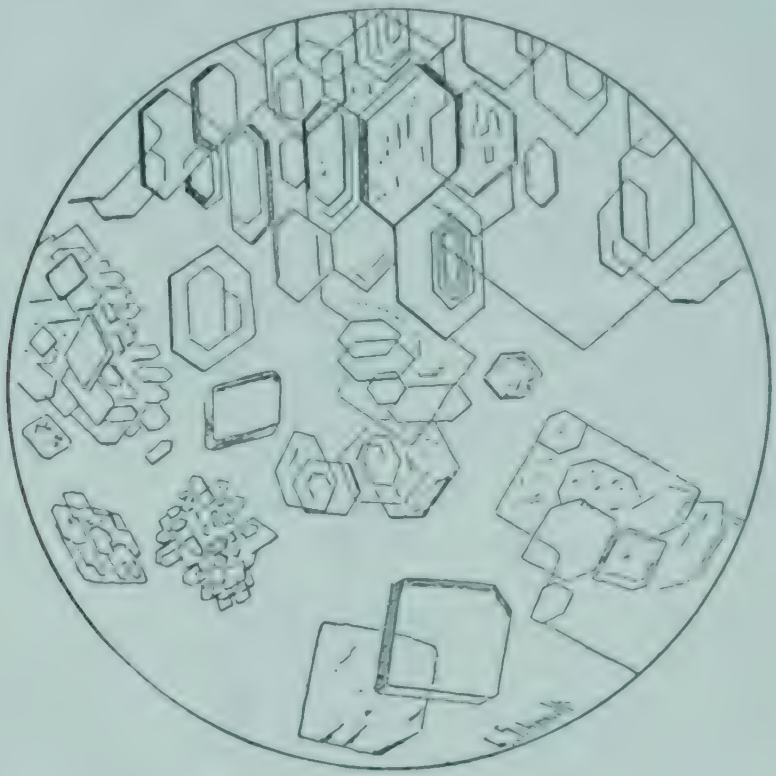


FIG. 218. UREA NITRATE.

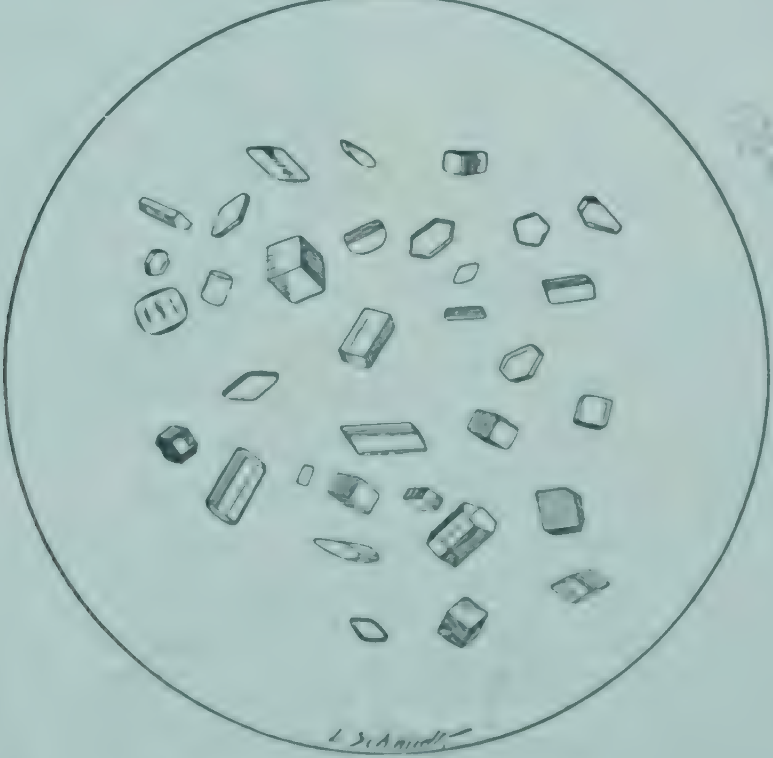


FIG. 219. UREA OXALATE.

Strong solutions of urea have a remarkable solvent effect upon proteins such as coagulated protein, and upon starch and other substances.

EXPERIMENTS ON UREA

1. *Isolation from the Urine.* Evaporate 200 ml. of urine in a casserole or evaporating dish over a free flame until the volume is reduced to about 25 ml. Transfer the container to a steam or water bath and continue the evaporation until the residue is semisolid, spreading the material over the sides of the dish to aid in removing as much water as possible. Add 50 ml. of acetone to the residue and stir thoroughly while the acetone is kept gently boiling on a previously heated water bath (with the flame turned out) or steam bath. Do not allow more than one-quarter of the acetone to boil off. Because of the inflammable and poisonous nature of acetone vapor, this and further operations should be conducted in the hood and in the absence of a free flame. Filter the hot extract quickly through a small dry filter into a dry 100-ml. beaker. Repeat the extraction of the residue once more with acetone, adding the filtrate to the first portion. Concentrate the combined extracts to a volume of 25 ml. on the steam bath or by placing the beaker in hot water. Chill the concentrated solution with cold water, cover, and allow to stand until crystallization occurs. Filter off the urea crystals, wash them with a little acetone, and allow to dry in air. Examine the crystals under the microscope and compare them with those shown in Fig. 217.

2. *Melting Point.* Determine the melting point of some urea crystals as follows: Into an ordinary melting-point tube, sealed at one end, introduce powdered urea. Fasten the tube to the bulb of a thermometer and suspend the bulb and its attached tube in a small beaker containing concentrated sulfuric acid. Gently raise the temperature of the acid by means of a low flame, stirring the fluid continually, and note the temperature at which the urea begins to melt.

3. *Crystalline Form.* Dissolve a crystal of pure urea in a few drops of 95 per cent alcohol and place 1 to 2 drops of the alcoholic solution on a microscopical slide. Allow the alcohol to evaporate spontaneously, examine the crystals under the microscope, and compare them with those reproduced in Fig. 217. Recrystallize a little urea from water in the same way and compare the crystals with those obtained from the alcoholic solution.

4. *Formation of Biuret.* Place a small amount of urea in a dry test tube and heat carefully in a low flame. The urea melts at 132° C. and liberates ammonia. Continue heating until the fused mass begins to solidify. Cool the tube, dissolve the residue in dilute sodium hydroxide solution, and add very dilute copper sulfate solution (see p. 171). The purplish-violet color is due to the presence of biuret which has been formed from the urea through the application of heat as indicated. The chemistry of this reaction is shown on p. 791.

5. *Urea Nitrate.* Prepare a concentrated solution of urea by dissolving a little of the substance in a few drops of water. Place a drop of this solution on a microscopical slide, add a drop of concentrated nitric acid, and examine under the microscope. Compare the crystals with those reproduced in Fig. 218.

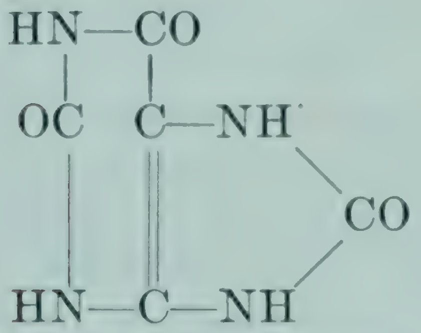
6. *Urea Oxalate.* To a drop of a concentrated solution of urea, prepared as described in Exp. 5, add a drop of a saturated solution of oxalic acid. Examine under the microscope and compare the crystals with those shown in Fig. 219.

7. *Decomposition by Sodium Hypobromite.* Into a mixture of 3 ml. of concentrated sodium hydroxide solution and 2 ml. of bromine water in a test tube introduce a crystal of urea or a small amount of concentrated solution of urea. Through the influence of the sodium hypobromite, NaOBr, the urea is decomposed and carbon dioxide and nitrogen are liberated. The carbon dioxide is absorbed by the excess of sodium hydroxide, while the nitrogen is evolved and causes the marked effervescence observed. This property forms the basis for one of the methods in common use for the quantitative determination of urea. Write the equation showing the decomposition of urea by sodium hypobromite.

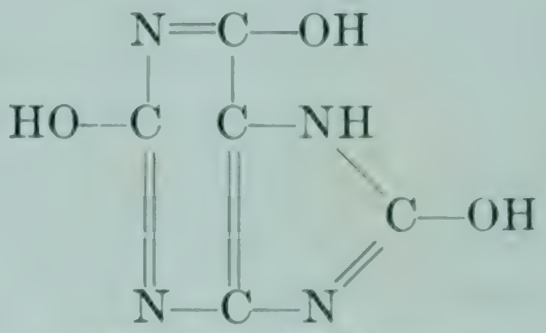
It is claimed that all ammonium compounds and all compounds containing the amino [$-NH_2$] group yield nitrogen when treated with hypobromite as in this test.

8. *Decomposition by Urease.* To 5 ml. of urea solution in a test tube add 1 ml. of urease solution or a little soybean or jack-bean powder. Allow the tube to stand for 10 minutes, heat the contents to boiling, holding moist red and blue litmus papers at the mouth of the tube. What do you observe? Note the odor. Explain.

URIC ACID



Keto (lactam) form



Enol (lactim) form

Uric acid is found in the urine normally to the extent of about 0.5 to 1.0 g. per 24 hours, but this amount is subject to wide variations, particularly under certain dietary and pathological conditions. On a purine-free diet the uric acid output may be 0.1–0.5 g. per day, whereas a high purine diet may yield a daily output of 2 g. Uric acid acts as a weak dibasic acid and forms two classes of salts, neutral and acid. The neutral potassium and lithium urates are the most easily soluble of the alkali salts; the ammonium urate is difficultly soluble. The acid salts are more insoluble and form the major portion of the sediment which separates upon cooling the concentrated urine; the alkaline earth urates are very insoluble. Ordinarily uric acid occurs in the urine in the form of urates and upon acidifying the liquid the uric acid is liberated and deposits in crystalline form.

Uric acid is closely related to the purine bases as may be seen from a comparison of its structural formula with those of the purine bases given on p. 202. According to the purine nomenclature, it is designated 2,6,8-trioxypurine. Uric acid forms the principal end product of the nitrogenous metabolism of birds and scaly reptiles; in the human organism it occupies a position quantitatively inferior to urea, ammonia, and creatinine.

In man, uric acid probably results principally from the destruction of nuclear or other purine material, ingested as food or from the disintegrating cellular matter of the organism. The uric acid resulting from the diet

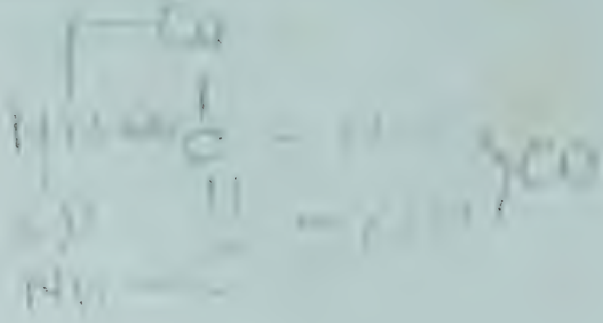
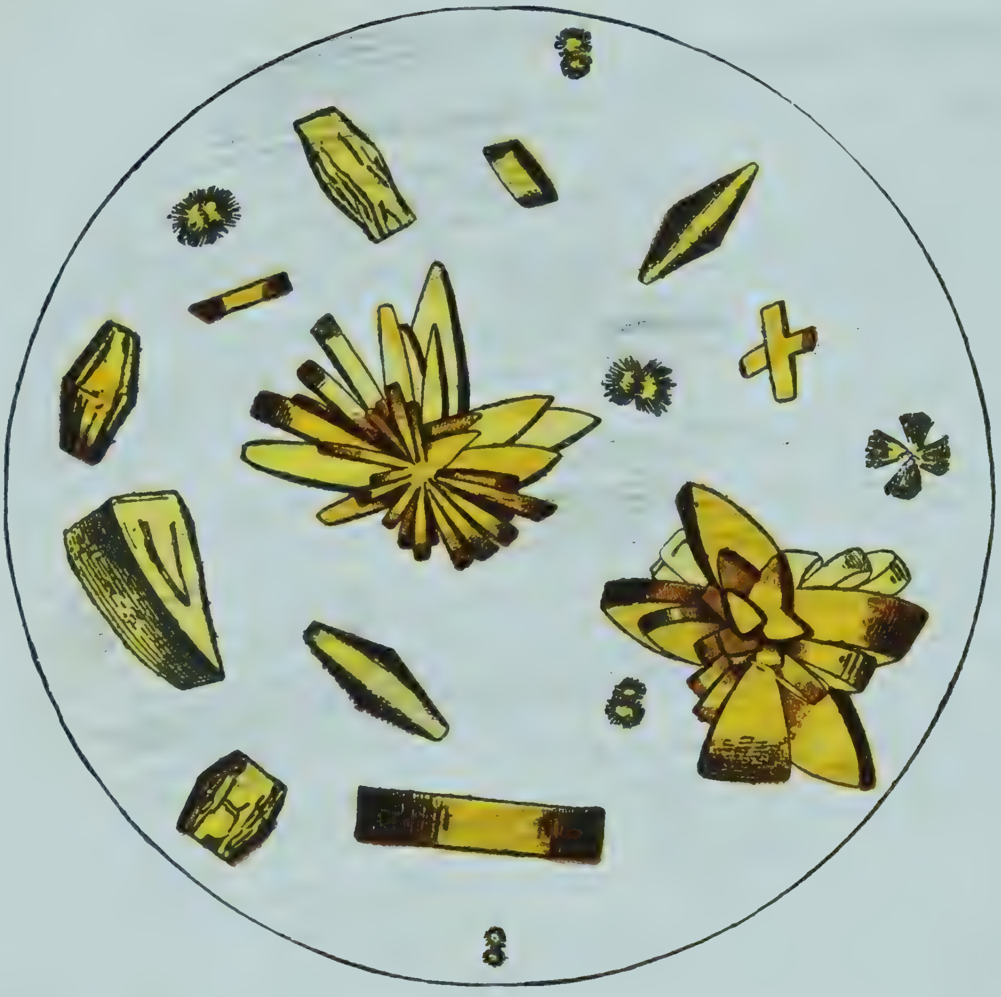
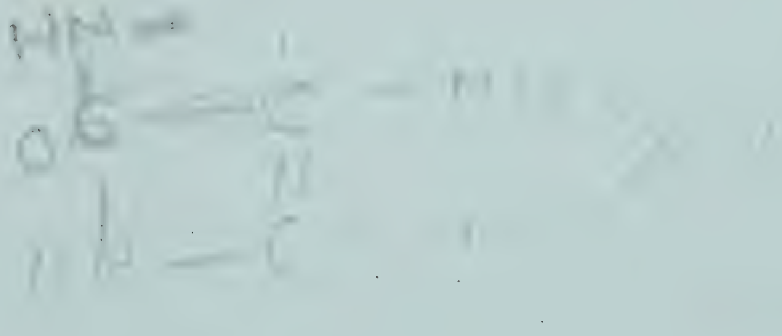


PLATE III



Uric Acid Crystals, Normal Colors.
(From Purdy, after Peyer.)



was formerly said to be of *exogenous* origin, whereas the product of cellular catabolism was said to be of *endogenous* origin. It is now known that metabolic activities cannot be properly explained on such a simple basis. However, the terms endogenous and exogenous are nevertheless frequently employed. Folin demonstrated that, following a pronounced decrease in the amount of protein metabolized, the absolute quantity of uric acid is decreased, but that this decrease is relatively smaller than the decrease in the total nitrogen excretion and that the percentage of the uric acid nitrogen, in terms of the total nitrogen, is therefore decidedly increased.

The enzymatic conversion of the purines adenine and guanine to the intermediates hypoxanthine and xanthine, and of the latter to uric acid by means of xanthine oxidase, is discussed in Chapter 7, "Nucleic Acids and Nucleoproteins." With the exception of man, the higher apes, and the Dalmatian dog, mammalia carry the conversion one step farther; i.e., to allantoin, through the action of the enzyme uricase. Despite the absence of a typical uricase from human tissues, there is no question but that the human organism has considerable ability to destroy uric acid, by mechanisms not known. Thus uric acid excretion represents a balance between the rate of production and the rate of destruction. From experiments on dogs, Mann and associates drew the conclusion that the destruction of uric acid depends on the presence of the liver. The extirpation of the liver causes an accumulation of uric acid in the blood and tissues or an increased elimination in the urine if renal activity is maintained. These experiments also indicate that the liver is of considerable importance in the general metabolism of the purines.

Using the isotope technique it has been shown[2] that orally administered uric acid is "extensively degraded" to urea, whereas intravenously administered uric acid is excreted essentially unchanged. This latter finding is in agreement with the reported absence of uricase in human tissues.

In birds the formation of uric acid is analogous to the formation of urea in man. In these organisms it is derived principally from the protein material of the tissues and the food and is formed through a process of synthesis which occurs for the most part in the liver; a comparatively small fraction of the total uric acid excretion of birds may result from nuclear material.

When pure, uric acid may be obtained as a white, odorless, and tasteless powder, which is composed principally of small, transparent, crystalline rhombic plates. Uric acid as it separates from the urine is invariably pigmented, and crystallizes in a large variety of characteristic forms, e.g., dumbbells, wedges, rhombic prisms, irregular rectangular or hexagonal plates, whetstones, prismatic rosettes, etc. Uric acid is insoluble in alcohol and ether, soluble with difficulty in boiling water (1:1800) and practically insoluble in cold water (1:39,480, at 18° C.). It is soluble in alkalies, alkali carbonates, boiling glycerol, concentrated sulfuric acid, and in certain organic bases such as ethylamine and piperidine. It is claimed that the uric acid is held in solution in the urine by the urea and disodium hydrogen phosphate present. Uric acid possesses the power of reducing cupric hydroxide in alkaline solution and may thus lead to an erroneous conclusion in testing for sugar in the urine by means of Fehling's or Trommer's test. A white precipitate of cuprous urate is formed if only a small amount of cupric hydroxide is present, but if enough of the copper salt is present the characteristic red or brownish-red precipitate of cuprous oxide is obtained. Uric acid does not possess the power of reducing bismuth in alkaline solution and therefore does not interfere in testing for sugar in the urine by means of Boettger's or Nylander's tests.

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[2] Geren, Bendich, Bodansky, and Brown: *J. Biol. Chem.*, **183**, 21 (1950).

In addition to being a constant urinary constituent uric acid is present in small amounts in normal human blood as well as in the blood of birds. It is also normally present in the brain, heart, liver, lungs, pancreas, and spleen.

Pathologically, the excretion of uric acid is subject to wide variations, but the experimental findings are rather contradictory. It may be stated with certainty, however, that in leukemia, because of the destruction of nuclear material, the uric acid output is increased absolutely as well as relatively to the urea output; under these conditions the ratio between the uric acid and urea may be as low as 1:9, whereas the normal ratio, as we have seen, is 1:50 or higher. An actual output of 12 g. of uric acid per day has been reported in leukemia.

In gout the kidney is said to lose the power of properly eliminating uric acid and it collects in the blood in abnormally high concentration. This is accompanied by the deposition of uric acid in the joint cartilages and especially in the joint of the big toe. Tests have been reported[3] which "minimize the role of dietary purine in the production of overt gout."

The uric acid content of the urine is of importance in relation to the formation of uric acid calculi. The administration of alkali carbonates and citrates, or the feeding of base-forming foods, by decreasing the acidity of the urine, increases its solvent power for uric acid and decreases the liability of formation of this type of calculus.

EXPERIMENTS ON URIC ACID

1. *Isolation from the Urine.* Place about 200 ml. of filtered urine in a beaker, render it acid with 2 to 10 ml. of concentrated hydrochloric acid, stir thoroughly, and stand the vessel in a cold place for 24 hours. Examine the pigmented crystals of uric acid under the microscope and compare them with those shown in Fig. 220 and Plate III.

2. *Crystalline Form of Pure Uric Acid.* Place about 100 ml. of water in a small beaker, render it distinctly alkaline with potassium hydroxide solution, and add a small amount of pure uric acid, stirring continuously. Cool the solution, render it distinctly acid with hydrochloric acid, and allow it to stand in a cool place for crystallization. Examine the crystals under the microscope and compare them with those reproduced in Fig. 220.

3. *Murexide Test.* To a small amount of pure uric acid in a small evaporating dish add 2 to 3 drops of concentrated nitric acid. Carefully evaporate to dryness on a water bath or over a very low flame. A red or yellow residue

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[3] Stetten: *Bull. N. Y. Acad. Med.*, 28, 664 (1952).

remains which turns purplish-red after the dish has been cooled and a drop of very dilute ammonium hydroxide has been added. The color is due to the formation of murexide. If potassium hydroxide is used instead of ammonium hydroxide, a purplish-violet color due to the production of the potassium salt is obtained. The color disappears upon warming; with certain related compounds (purine bases) the color persists under these conditions. This is a valuable test for the detection of uric acid calculi.

In this reaction the uric acid is oxidized to dialuric acid and alloxan. These two substances condense to form alloxantin. This alloxantin reacts with ammonium hydroxide to form purpuric acid. The purple color is due to the formation of ammonium purpurate or murexide.

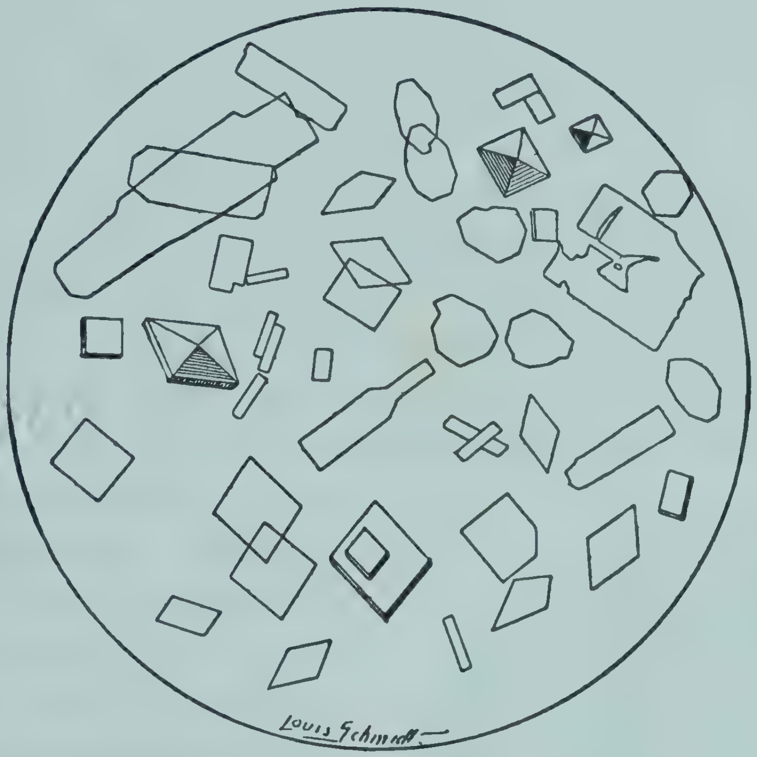
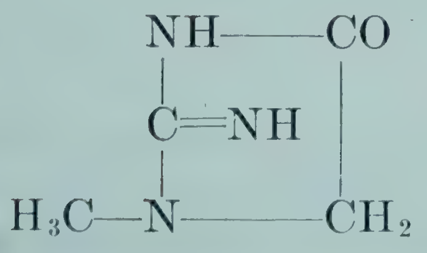


FIG. 220. PURE URIC ACID.

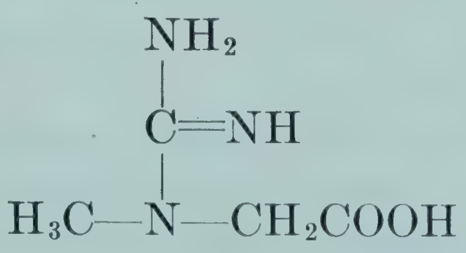
4. *Phosphotungstic Acid Reaction* (*Folin*). To 20 ml. of saturated sodium carbonate solution in a small beaker add a small amount of uric acid. Stir the solution until the uric acid has dissolved, then add 1 ml. of Folin's uric acid reagent (see p. 560). A blue color results.

5. *Silver Reduction Test* (*Schiff*). Dissolve a small amount of pure uric acid in sodium carbonate solution and transfer a drop of the resulting mixture to a strip of filter paper saturated with silver nitrate solution. A yellowish-brown or black coloration due to the formation of reduced silver is produced. It is claimed that chlorides interfere with this test.

CREATININE



CREATINE



$$H_3C-N(-CH_2COOH)-C(=NH)-NH_2$$

Creatinine. Creatinine is the anhydride of creatine (methylguanidinoacetic acid) and is a constant constituent of normal human urine. Under normal conditions about 1 to 1.8 g. of creatinine are excreted by an adult man in 24 hours. The exact amount of creatinine excreted under ordinary circumstances depends in part upon the nature of the diet, since any creatinine in the diet is excreted unchanged in the urine. Foods such as meat and fish contain significant amounts of performed creatinine, particularly after cooking. Creatinine excretion decreases somewhat in starvation. The absolute amount of creatinine eliminated in the urine *on a creatinine-free diet* is practically a constant quantity for a given individual and is independent of quantitative changes in the total amount of nitrogen eliminated under these conditions. The "creatinine coefficient," which is the daily excretion of creatinine in mg. per kg. of body weight,[4] is an index of this constancy of creatinine elimination. Endogenous creatinine is apparently the result of some special process of normal metabolism which takes place at a constant rate and almost certainly involves body creatine, since creatine can be converted in the body to creatinine. For example, when animals are given tagged creatine the isotopic ratios of tissue creatine and urine creatinine are similar. The conversion of creatinine to creatine in the body does not occur.

Very little that is important is known regarding the excretion of creatinine under pathological conditions. The creatinine content of the urine is said to be increased in conditions associated with increased tissue catabolism, such as fever. The output of creatinine is decreased in disorders associated with muscular atrophy and muscular weakness. The greater part of the data relating to the variation of the creatinine excretion under pathological conditions are not of much value since in nearly every instance the diet was not sufficiently controlled to permit the collection of reliable data.

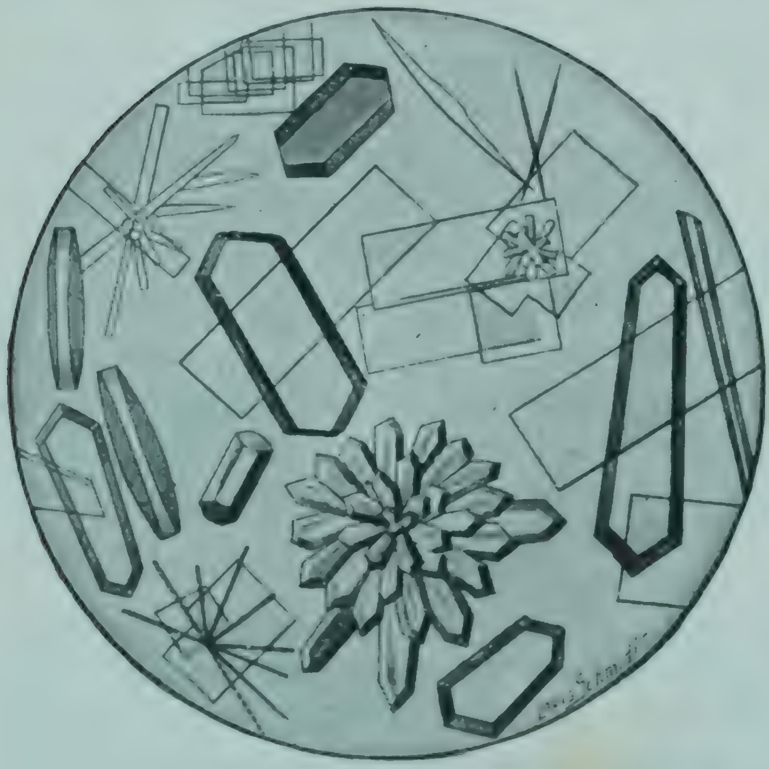


Fig. 221. Creatinine.

According to Mann and associates the changes in the blood creatinine after removal of the liver depend entirely on the kidneys. If the kidneys are active the blood creatinine does not change. If the kidneys are removed as well, or the animal becomes anuric, the creatinine increases in the blood at the same rate as when only the kidneys are removed. The creatine behaves in a similar manner. The liver would therefore appear to take no specific part in the creatinine-creatine relationship.

Creatinine crystallizes in colorless, glistening, monoclinic prisms (Fig. 221) which are soluble in about 12 parts of cold water; they are more soluble in warm water and in warm alcohol. It forms salts only with strong mineral acids. One of the most important and interesting of the compounds of creatinine is creatinine-zinc chloride, $(C_4H_7N_3O)_2ZnCl_2$.

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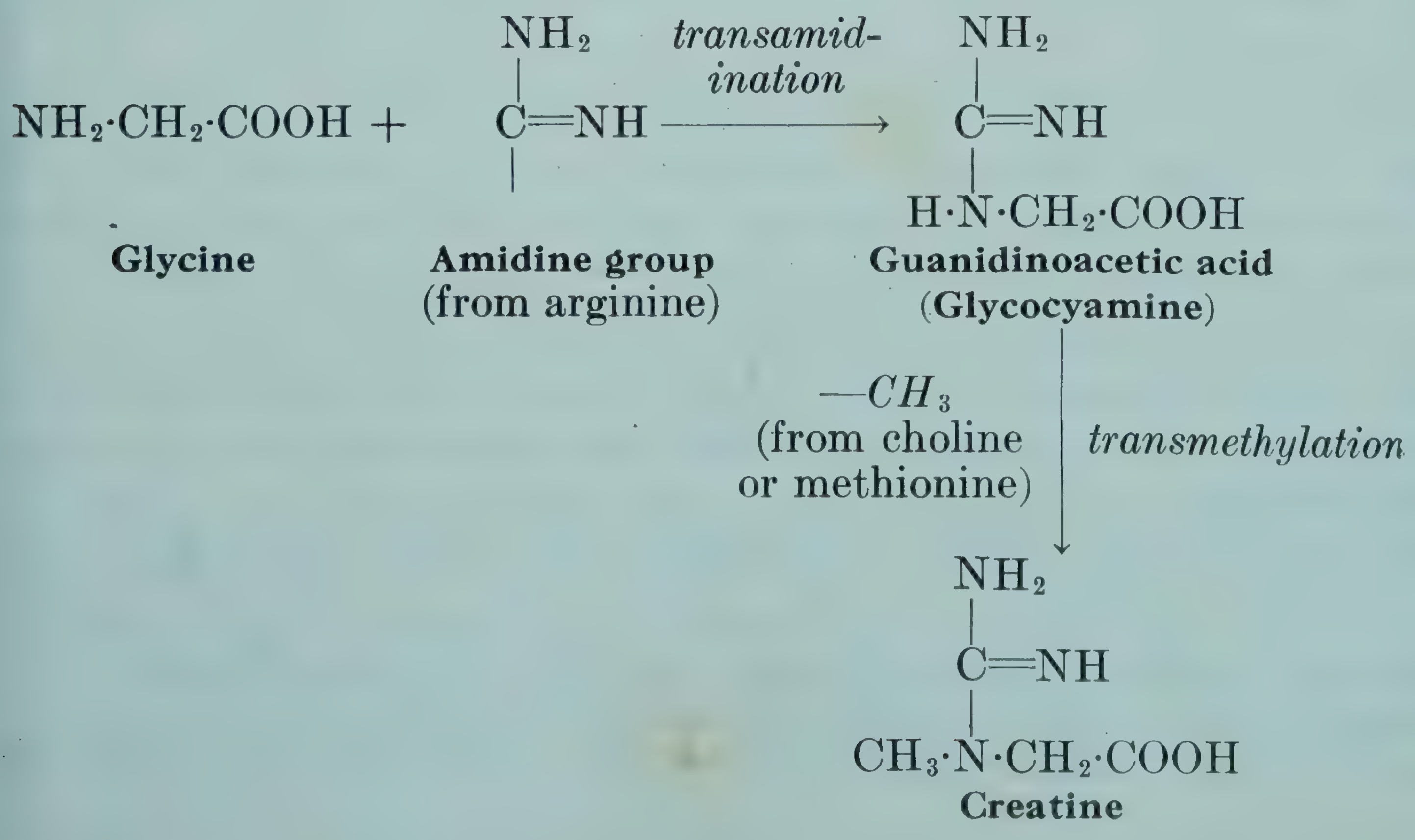
[4] Shaffer designates as the "creatinine coefficient" the excretion of creatinine *nitrogen* in mg. per kg. of body weight.

which is formed from an alcoholic solution of creatinine upon treatment with zinc chloride in acid solution. Creatinine has the power of reducing cupric hydroxide in alkaline solution and of forming an insoluble cuprous-creatinine salt, and in this way may interfere with the determination of sugar in the urine. Creatinine does not reduce alkaline bismuth solutions and therefore does not interfere with Nylander's and Boettger's tests.

Creatine. Creatine occurs in very small amounts in the urine of normal adults but is found in larger amounts in that of children and of pregnant women. The amount is increased in fasting, in pregnancy, and after high water ingestion. Increased amounts have been found associated with malnutrition and disintegration of muscular tissue, in fever, and in carcinoma of the liver. The presence of readily detectable amounts of creatine in the urine is called *creatinuria.*

Creatine in the diet does not normally appear as either extra creatinine or creatine in the urine, being apparently incorporated by the body in its total supply of creatine, where it undergoes metabolism at the same rate as that already present. The fact that ingestion of a gram or so of creatine does not normally lead to any change in urinary creatinine or creatine is used clinically in the so-called *creatine tolerance test;* in certain diseases involving muscular tissue chiefly, ingestion of creatine is followed by the appearance of extra creatine in the urine, i.e., the patient's tolerance for creatine is decreased. Creatine occurs in all tissues, but in much greater amount in the muscular tissues than elsewhere. The average adult human body contains about 100 g. of creatine, largely if not entirely in the form of phosphocreatine.

Creatine is synthesized in the animal body. The mechanism of this synthesis resisted elucidation for many years, finally yielding to the application of isotopes and the tissue-slice technique, through the work of Schoenheimer, du Vigneaud, Borsook, and their collaborators. While other reactions may not be entirely excluded, these investigators have shown that the major processes in the biological synthesis of creatine are as follows:



Thus the synthesis involves the amino acids glycine and arginine in the reaction of transamidination, and a source of labile methyl groups in the transmethylation reaction; the latter may involve either the amino acid methionine or the substance choline. In this connection it is of interest that while glycine is readily synthesized by man, it cannot be synthesized by the chick and is ordinarily required in the diet but may be replaced there by creatine. Of interest also is the clinical observation that in myasthenia gravis the feeding of glycine leads to an increased excretion of creatine. The intermediate compound guanidinoacetic acid is found in normal urine to the extent of about 20 mg. per day.

Creatine is fairly soluble in water but the aqueous solution is unstable, the creatine being gradually transformed to its anhydride creatinine by ring closure after the splitting off of water. This process is accelerated by heat and acid, and this is the basis of the usual procedures for the detection or determination of creatine, the creatine being converted into creatinine and tested for as the latter compound. There is no satisfactory direct test reaction for creatine itself.

EXPERIMENTS ON CREATININE AND CREATINE

1. *Preparation of Pure Creatinine from Urine* (*Folin-Benedict*). To 10 liters of undecomposed urine in a large precipitating jar add with stirring a hot solution of 180 g. of picric acid in 450 ml. of boiling alcohol. Allow to stand overnight and syphon off the supernatant fluid. Pour the residue upon a large Buchner funnel, drain with suction, wash once or twice with cold saturated picric acid, and suck dry. Treat the dry or nearly dry picrate in a large mortar or evaporating dish with enough concentrated HCl to form a moderately thin paste (about 60 ml. of acid for each 100 g. of picrate) and stir the mixture thoroughly with the pestle for 3 to 5 minutes. Filter with suction on a hardened paper, and wash the residue twice with enough water to cover it, sucking as nearly dry as possible each time. Transfer the filtrate to a large flask and neutralize with an excess of solid magnesium oxide (the "heavy" variety is best). Add this oxide in small portions with cooling of the flask under running water between the additions. Neutralization of the acid will be indicated by a bright yellow color of the mixture, or litmus paper may be used to test it. Filter with suction. Wash the residue twice with water. Immediately add a few ml. of glacial acetic acid to the filtrate to make it strongly acid. Neglecting any precipitate that may form, dilute the solution with about 4 volumes of 95 per cent alcohol. After 15 minutes filter off the slight precipitate which forms. Treat the final filtrate with 30 to 40 ml. of 30 per cent zinc chloride. Stir and let stand overnight in a cool place. Pour off the supernatant liquid and collect the creatinine zinc chloride on a Buchner funnel, wash once with water, then thoroughly with 50 per cent alcohol, finally with 95 per cent alcohol and dry. A nearly white, light crystalline powder should be obtained. The yield should be 90 to 95 per cent of the original creatinine (usually about 1.5 to 1.8 g. of creatinine zinc chloride per liter of urine).

Recrystallize the creatinine zinc chloride by treating 10 g. with 100 ml. of water and about 60 ml. of normal sulfuric acid, heating the mixture until a clear solution is obtained. Add about 4 g. of purified animal charcoal, continue boiling for about a minute, filter with suction through a small Buchner funnel, pouring the filtrate back on the filter 3 or 4 times until it runs through perfectly colorless. Wash the residue with hot water and

transfer the total filtrate to a beaker and while hot treat with a little strong zinc chloride solution (3 ml.) and with about 7 g. of potassium acetate dissolved in a little water. After 10 minutes dilute with an equal volume of alcohol, and allow to stand in a cold place for some hours. Filter off the crystalline product and examine under a microscope (see Fig. 222). To remove the small amount of potassium sulfate which it contains stir up with its weight of water, filter, wash with a little water and then with alcohol. The preparation should be snow white. Yield, 85 to 90 per cent.

For the decomposition of the creatinine zinc chloride Gaebler suggests the following modification of the original procedure. Place 32 g. creatinine zinc chloride in a pressure bottle (a citrate of magnesia bottle will serve) and add 225 to 250 ml. of concentrated ammonia. Close the bottle and heat in a water bath at 70 to 80° C., shaking to effect solution. Cool quite rapidly to room temperature and then in a salt-ice bath. Pure creatinine crystallizes out. Filter. Wash with ice-cold ammonia, then with acetone, and dry. The yield should be 75 per cent (15 g. of creatinine). The product is perfectly pure and can be used as a standard in the quantitative determination of creatine and creatinine. See the chapters on quantitative analysis of urine and blood.

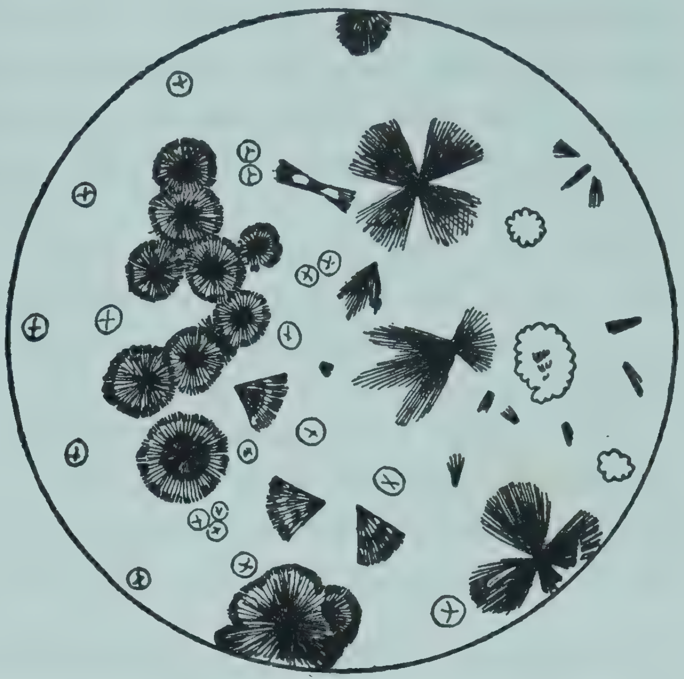


FIG. 222. CREATININE ZINC CHLORIDE (SALKOWSKI).

2. *Nitroprusside Test* (*Weyl*). Take 5 ml. of urine in a test tube, add a few drops of sodium nitroprusside, and render the solution alkaline with NaOH solution. A ruby-red color results which soon turns yellow. See Legal's test for acetone, Chapter 29.

3. *Nitroprusside–Acetic Acid Test* (*Salkowski*). To the yellow solution obtained in Weyl's test above, add an excess of acetic acid and apply heat. A green color results and is in turn displaced by a blue color. A precipitate of prussian blue may form.

4. *Picric Acid Reaction* (*Jaffé*). Place 5 ml. of urine in a test tube, add an aqueous solution of picric acid, and render the mixture alkaline with NaOH solution. A red color is produced which turns yellow if the solution be acidified. Glucose gives a similar red color but only upon the application of heat. This color reaction observed when creatinine in alkaline solution is treated with picric acid is the basic principle of Folin's colorimetric method for the quantitative determination of creatinine (see Chapter 31) and is due to the formation of a red tautomer of creatinine picrate. The production of this tautomer is "dependent upon the formation of a salt, a keto-enol change within the creatinine molecule, and a change in the picric acid molecule involving the hydrogens in the meta positions and, probably, all three nitro groups."

5. *Preparation of Creatine.* Creatine may be prepared from creatinine zinc chloride by decomposition with calcium hydrate, the process being one of hydrolysis (Benedict).

100 g. of creatinine zinc chloride are treated with about 700 ml. of water in a large casserole and the mixture heated to boiling; 150 g. of pure powdered calcium hydrate are then added, with stirring, and the mixture boiled gently for 20 minutes (with occasional stirring). The hot mixture is then filtered with suction, the residue being washed with hot water. The filtrate is then treated with hydrogen sulfide gas for a few minutes and poured through a folded filter to remove the zinc. The filtrate is acidified by the addition of about 5 ml. of glacial acetic acid and boiled down rapidly to a volume of about 200 ml. This solution is allowed to stand overnight, preferably in a cool place. The next day the crystallized creatine is filtered off with suction, washed with a very little cold water, and then thoroughly washed with alcohol and dried. This product is then recrystallized by dissolving in about 7 times its weight of boiling water and allowing the solution to cool slowly and stand for some hours. This product should be perfectly pure creatine. If necessary it can be recrystallized with very little loss. The crystallized product should be filtered off, washed with alcohol and ether, and dried in air for about half an hour. Thus obtained, the creatine contains water of crystallization which it loses very readily upon exposure to air. To prepare creatine which can be weighed with absolute exactness it is necessary to dehydrate this product by heating for some hours at about 95° C.

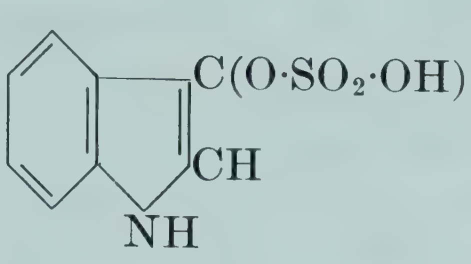
The yield in this process is about 18 g. of recrystallized creatine, and about 55 g. of creatinine zinc chloride recovered. Longer boiling with lime does not bring about a greater yield, for after the 20-minute point creatine is decomposed almost exactly as fast as it is formed.

Examine the crystals of creatine under the microscope and compare with illustration in Chapter 10, "Muscular Tissue," in which may be found other creatine tests.

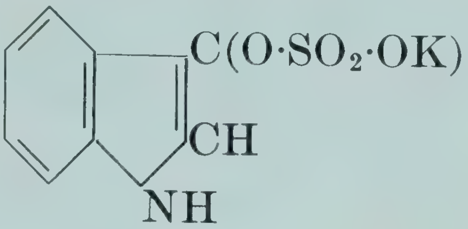
ETHEREAL SULFATES

The most common ethereal sulfates found in the urine are phenolsulfuric acid, *p*-cresolsulfuric acid, indoxylsulfuric acid, and skatoxylsulfuric acid. Pyrocatecholsulfuric acid also occurs in traces in human urine. The total output of ethereal sulfuric acid (as S) varies ordinarily from 0.04 to 0.1 g. for 24 hours and comprises 5 to 15 per cent of the total sulfur. In health the ratio of ethereal plus neutral sulfate to inorganic sulfate is about 1:10. These ethereal sulfuric acids originate in part from the phenol, cresol, indole, and skatole formed in the putrefaction of protein material in the intestine. The phenol passes to the liver where part of it is conjugated to form phenol potassium sulfate and appears in this form in the urine, whereas the indole and skatole undergo a preliminary oxidation to form indoxyl and skatoxyl respectively before their conjugation and elimination. (See Chapter 20.)

It was formerly generally considered that each of the ethereal sulfuric acids was formed principally in the putrefaction of protein material in the intestine and that therefore a determination of the total ethereal sulfuric acid content of the urine was an index of the extent to which these putrefactive processes were proceeding within the organism. Folin, however, showed that the ethereal sulfuric acid content of the urine did *not* afford an index of the extent of intestinal putrefaction, since these compounds arise only in part from putrefactive processes. He claimed that the ethereal sulfuric acid excretion represented a form of sulfur metabolism which is more in evidence when the diet contained little or no protein. The ethereal sulfuric acid content of the urine diminishes as the total sulfur content diminishes, but the percentage decrease is much less. Therefore, relative to the total sulfuric acid content, the ethereal sulfuric acid content is increased, although the total sulfuric acid content is diminished. The indoxylsulfuric acid (indican) content of the urine does not originate to any degree from the tissue catabolism of protein material but arises in great part from the action of intestinal putrefactive organisms on tryptophan (see Chapter 20). The excretion of indoxylsulfuric acid



which occurs in the urine as the potassium salt



may *alone* be taken as a rough index of the extent of putrefactive processes within the intestine and is clinically the most important of the ethereal sulfuric acids. Under normal conditions, from 10 to 20 mg. of indican are excreted per day. The variations are due mainly to diet, a high meat diet causing an increase and a carbohydrate diet a decrease. Pathologically, the greatest increases are found in disorders involving increased putrefaction and stagnation of intestinal contents. Bacterial decomposition of body protein as in gangrene, putrid pus formation, etc., gives rise to an increased indican excretion.

Phenols are excreted chiefly in the conjugated form. The phenol output tends to vary directly but not proportionally with the protein ingestion. The total phenol excretion of normal men on an ordinary mixed diet averages around 0.2 g. per day.

TESTS FOR INDICAN[5]

1. *Jaffé's Test*. Nearly fill a test tube with a mixture composed of equal volumes of concentrated HCl and the urine under examination. Add 2 to 3 ml. of chloroform and a few drops of a calcium hypochlorite solution, place the thumb over the end of the test tube and rock the tube back and forth, inverting at least 10 times. The chloroform is more or less colored, according to the amount of indican present. Ordinarily a blue color due to the forma-

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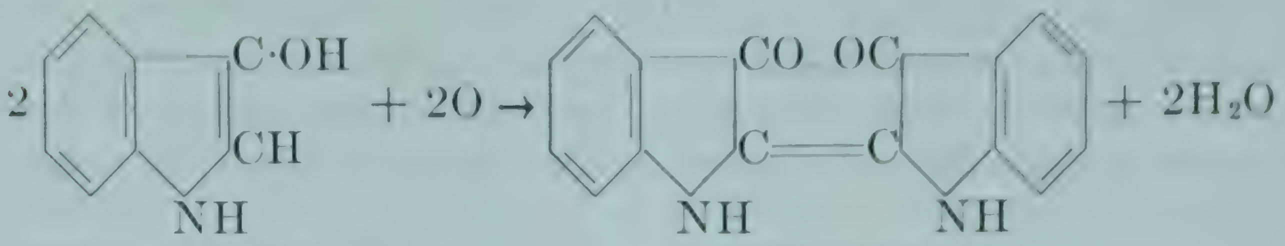
[5] The urine should always be examined fresh if this is possible. In any event formaldehyde should never be used as a preservative for urines which are to be examined for indican by means of any test involving hypochlorite or potassium permanganate. The formaldehyde through its reducing power lowers the oxidizing efficiency of the mixture. The formation of formic acid from the aldehyde may also interfere.

tion of indigo blue is produced; less frequently a red color due to indigo red may be noted.

Repeat this test on some of this same urine to which formaldehyde has been added. Is there any variation in the reaction from what you previously obtained?

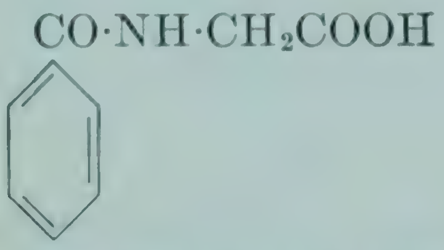
The following represents the reaction:



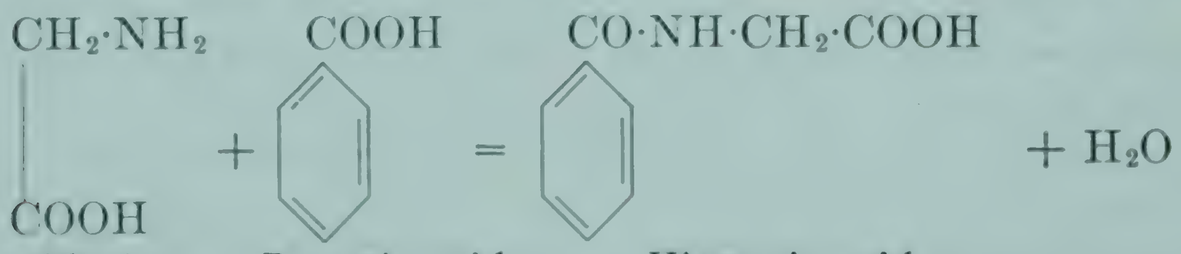
Indoxyl, C_8H_7NO **Indigo blue**

2. *Obermayer's Test*. Nearly fill a test tube with a mixture composed of equal volumes of Obermayer's reagent[6] and the urine under examination. Add 2 to 3 ml. of chloroform, place the thumb over the end of the test tube, and rock the tube back and forth, inverting at least 10 times. How does this compare with Jaffé's test?

HIPPURIC ACID



This acid occurs normally in the urine of both carnivora and herbivora, but it is much more abundant in the urine of the latter. It is formed by the union of benzoic acid with glycine according to the following reaction:



Glycine **Benzoic acid** **Hippuric acid**

In the dog this conjugation takes place exclusively in the kidneys, but in man and in the rabbit it is probable that the liver is the main site of this synthesis. Clinically the ability of an individual to synthesize hippuric acid after administration of a test dose of benzoate is used as an index of liver function. Glycine is readily produced by the body from the hydrolysis of dietary or tissue protein, or may be synthesized from certain other amino acids, e.g., serine. Benzoic acid originates ordinarily either from intestinal putrefaction, i.e., from the abnormal metabolism of tyrosine and phenylalanine, or from the food. Many vegetables, fruits, and grasses contain small quantities of preformed benzoic acid and larger amounts of quinic acid and other compounds which in the body are converted to benzoic acid. It has been found that approximately 2 g. of benzoic acid are excreted as hippuric acid after eating 250 g. of prunes. The average excretion of hippuric acid by an adult man for 24 hours under normal conditions is about 0.7 g. Hippuric acid crystallizes in needles or rhombic prisms (see Fig. 223), the particular form depending upon the rapidity of crystallization. Pure hippuric acid melts at 187° C. It is easily soluble in alcohol or hot water. It is sufficiently soluble in ether to allow its extraction from aqueous solution with this solvent. (For the quantitative determination of hippuric acid, see Chapter 31.)

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[6] See Appendix.

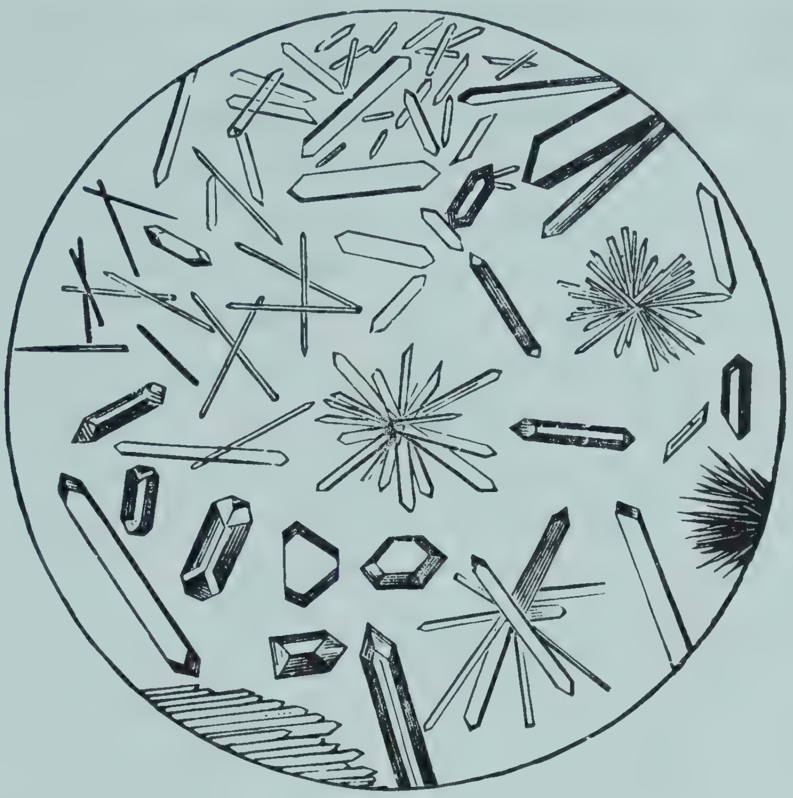


FIG. 223. HIPPURIC ACID.

EXPERIMENTS ON HIPPURIC ACID

1. Separation from the Urine. **(See Chapter 31.)**

2. Melting Point. **Determine the melting point of the hippuric acid prepared in the above experiment (see p. 793).**

3. Formation of Nitrobenzene (Lücke's Reaction). **To a little hippuric acid in a small porcelain dish add 1 to 2 ml. of concentrated HNO_3 and evaporate to dryness on a water bath. Transfer the residue to a dry test tube, apply heat, and note the odor of nitrobenzene (resembling that of oil of bitter almonds).**

4. Sublimation. **Place a few crystals of hippuric acid in a dry test tube and apply heat. The crystals are reduced to an oily fluid which solidifies in a crystalline mass upon cooling. When stronger heat is applied the liquid assumes a red color and finally yields a sublimate of benzoic acid and the odor of hydrocyanic acid.**

OXALIC ACID

$$\begin{array}{c}\mathrm{COOH}\\ |\\ \mathrm{COOH}\end{array}$$

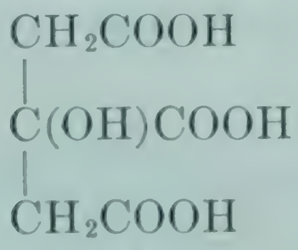
Oxalic acid is a constituent of normal urine, about 15 to 20 mg. being eliminated in 24 hours. It separates out from neutral or alkaline urine as the insoluble crystalline *calcium oxalate*, in the form of either dumbbells or octahedra, usually the latter (see Fig. 230). Many urinary calculi consist largely of calcium oxalate. When ingested oxalic acid is eliminated, at least in part, unchanged. Since many common articles of diet, e.g., asparagus, apples, cabbage, grapes, lettuce, rhubarb, spinach, tomatoes, etc., contain oxalates (or precursors) it seems probable that they are responsible for most of the oxalic acid found in the urine. The ingestion of rhubarb leaves has caused oxalic acid poisoning. They contain about 1.2 per cent of the acid. Spinach contains 0.8 to 0.9 per cent. The eating of these vegetables tends to lessen the supply of calcium in the body. Other green vegetables such as lettuce, celery, endive, asparagus, broccoli, Brussels sprouts, and cabbage contain very small amounts of oxalic acid—generally less than 0.1 per cent. There is also experimental evidence that part of the oxalic acid of the urine is formed within the organism in the course of protein and fat metabolism. It has also been suggested that oxalic acid may arise from an incomplete combustion of carbohydrates, especially under certain abnormal conditions. In this connection it is of interest that oxalic acid is one of the end products of *in vitro* oxidation of ascorbic acid. Pathologically, urinary oxalic acid is increased in diabetes mellitus, in organic diseases of the liver, and in various other conditions which are accompanied by a derangement of the oxidative mechanism. An abnormal increase in oxalic acid excretion is termed oxaluria and may be unaccompanied by any other apparent symptom.

Oxaluric acid ($NH_2{\cdot}CO{\cdot}NH{\cdot}CO{\cdot}COOH$) is occasionally found in traces in normal human urine. On hydrolysis it yields oxalic acid and urea.

EXPERIMENT ON OXALIC ACID

Precipitation of Calcium Oxalate. **Place 200 to 250 ml. of urine in a beaker, add 5 ml. of a saturated solution of calcium chloride, make the urine slightly acid with acetic acid, and stand the beaker in a cool place for 24 hours. Examine the sediment under the microscope and compare the crystalline forms with those shown in Fig. 230, p. 855.**

CITRIC ACID



$$\begin{array}{l} CH_2COOH \\ | \\ C(OH)COOH \\ | \\ CH_2COOH \end{array}$$

Citric acid is a normal urinary constituent, the 24-hour excretion ranging from about 300 to 1500 mg. Several factors have been noted to influence the excretion rate. It is enhanced in alkaline urine,[7] by estrogens,[8] and with increased urinary excretion of calcium.[9] Urinary citric acid excretion is reduced in acid urine and by androgens. The effect of endogenous estrogens in augmenting urinary citric acid excretion is seen in the characteristic curve of the normal menstrual cycle, with the lowest excretion during menstruation followed by a mid-menstrual peak and plateau and a return to the lower menstrual levels just before the next menstrual period. Since the citrate ion forms a soluble weakly ionized complex with calcium, an effort has been made to apply this augmenting effect of estrogens to the management of renal stones containing calcium. This application of estrogens is liable to be ineffective owing to the frequent association of urinary infections with organisms which destroy citric acid and is further limited in usefulness by its interference with menstrual cycles and with libido in the male. An enzyme, citrogenase, found in the kidney, heart, and liver can form citric acid by condensation of pyruvic and oxalacetic acids. A marked increase in urinary citric acid following hepatectomy suggests that the liver has a function in its intermediary metabolism.

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[7] Östberg: *Skand. Arch. Physiol.*, **62**, 81 (1931).

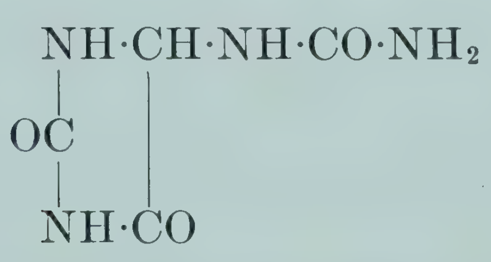
[8] Shorr, Bernheim, and Taussky: *Science*, **95**, 606 (1942).

[9] Shorr: *J. Urology*, **53**, 507 (1945).

NEUTRAL SULFUR COMPOUNDS

Under this head may be classed such substances as cystine, methyl mercaptan, ethyl sulfide, thiocyanates, taurine derivatives, etc. The sulfur content of the compounds just enumerated is generally termed unoxidized or neutral sulfur in order that it may not be confused with the acid or oxidized sulfur which occurs in the inorganic sulfuric acid and ethereal sulfuric acid forms, although this distinction is admittedly inexact. Ordinarily the neutral sulfur content of normal human urine is 5 to 25 per cent of the total sulfur content (see "Partition of Urinary Nitrogen and Sulfur," Chapter 31). The actual amount excreted may be 0.08 to 0.16 g. per day, calculated as S. Its origin is mainly endogenous. The excretion is fairly constant for any given individual in spite of dietary changes. In cystinuria, or in certain degenerative diseases, the amount is increased. (See p. 168 for test for cystine and cysteine sulfur.)

ALLANTOIN



Allantoin is found in the urine of practically all mammals including man. In human urine it occurs in very small amounts (5 to 15 mg. per day) whereas in all other mammals except anthropoid apes, and the Dalmatian coach dog, it is the principal end product of purine metabolism and may constitute 90 per cent or over of the total purine output. Allantoin is formed upon oxidation of uric acid by the enzyme uricase and the output is increased by the feeding of thymus or pancreas to lower animals. In the dog, according to Mann and associates, the liver is the sole seat of uric acid destruction, since after hepatectomy the uric acid excretion is equivalent to the allantoin output of the normal animal. When pure, allantoin crystallizes in prisms (Fig. 224), and when impure in granules and knobs.

EXPERIMENTS ON ALLANTOIN

1. *Separation from the Urine:*[10] *Meissner's Method.* Precipitate the urine with baryta water. Neutralize the filtrate carefully with dilute sulfuric acid, filter immediately, and evaporate the filtrate to incipient crystallization. Completely precipitate this warm fluid with 95 per cent alcohol (reserve the precipitate). Decant or filter and precipitate the solution by ether. Combine the ether and alcohol precipitates, and extract with cold water or hot alcohol; allantoin remains undissolved. Bring the allantoin into solution in hot water and recrystallize.

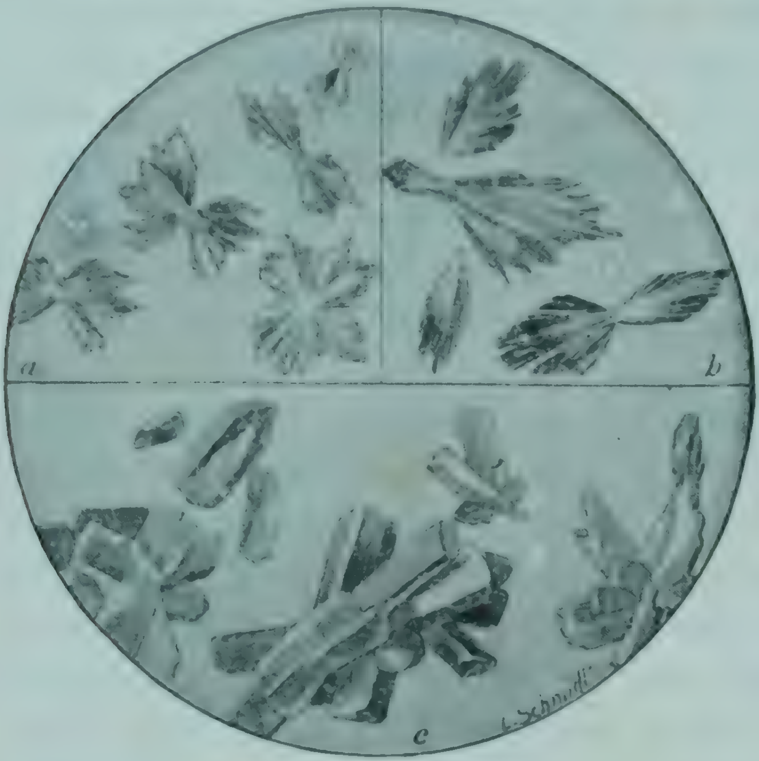


Fig. 224. Allantoin, from Cat's Urine.
a, *b*, Forms in which it crystallized from the urine; *c*, recrystallized allantoin.

2. *Preparation from Uric Acid.* Dissolve 4 g. of uric acid in 100 ml. of water rendered alkaline with potassium hydroxide. Cool and carefully add 3 g. of potassium permanganate. Filter, immediately acidulate the filtrate with acetic acid, and allow it to stand in a cool place overnight. Filter off the crystals and wash them with water. Save the wash water and filtrate, unite them, and after concentrating to a small volume stand away for crystallization. Now combine all the crystals and recrystallize them from hot water. Use these crystals in the experiments which follow.

3. *Microscopical Examination.* Examine the crystals made in Exp. 2 and compare them with those shown in Fig. 224.

4. *Furfural Test (Schiff).* Place a few crystals of allantoin on a test tablet or in a porcelain dish and add 1 to 2 drops of a concentrated aqueous solution of furfural and 1 to 2 drops of concentrated hydrochloric acid. Observe the formation of a yellow color, which turns to a light purple if allowed to stand. This test is given by urea but not by uric acid.

5. *Murexide Test.* Try this test according to the directions given on p. 796. Note that allantoin fails to respond.

AMINO ACIDS

Certain of these acids are always present in normal urine. The excretion of total amino acid nitrogen by a normal adult averages 0.4 to 1.0 g. per day or about 2 to 6 per cent of the total nitrogen. Free amino acid nitrogen (for methods of estimation, see Chapter 31) is considerably less than this, and ordinarily constitutes 0.5 to 1 per cent of the total nitrogen. The amount may be largely increased in disorders associated with tissue waste, e.g., typhoid, acidosis, pronounced atrophy of the liver, etc. After extirpation of the liver, urea formation ceases and amino acids accumulate in the blood or are excreted in the urine if renal activity is maintained. For tests on amino acids, see Chapter 4.

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[10] The urine of the dog after thymus, pancreas, or uric acid feeding may be employed

The availability of microbiological assay (see p. 1061) and paper chromatography (see p. 14) has made possible the detection and estimation of the individual amino acids present in normal and pathological urine. In one study on 18 normal male and female subjects on a normal diet, Woodson, *et al.*[10a] found most of the common amino acids present in the urine, in either the free state or in a combined form or both. The mean 24-hour excretion of free amino acids ranged from approximately 1 mg. for aspartic acid to 188 mg. for histidine; for the combined form, from 2 mg. for methionine to 315 mg. for glutamic acid. Those amino acids excreted predominantly in the combined form included aspartic acid, glutamic acid, proline, valine, and isoleucine. The nature of the combined form is not known; either peptides or conjugates are possibilities. No correlation could be established between the excretion of the various amino acids and differences in urine volume, total nitrogen, uric acid, creatinine, or ammonia content of the urines examined.

Dent has made extensive studies of urinary excretion of amino acids, using the techniques of paper chromatography to which he has made valuable contributions.[10b] Investigations have been reported on the general aminoaciduria characteristic of Fanconi's syndrome; on the excessive excretion of cystine, lysine, and arginine in cystinuria; and on other anomalies of amino acid metabolism.

AROMATIC OXYACIDS

Two of the most important of the oxyacids are *p-hydroxyphenylacetic acid,* and *p-hydroxyphenylpropionic acid.* They are products of the putrefaction of protein material and tyrosine is an intermediate stage in their formation. Both these acids for the most part pass unchanged into the urine, where they occur normally in very small amount. The content may be increased in the same manner as the phenol content, in particular by acute phosphorus poisoning. A fraction of the total aromatic oxyacid content of the urine is in combination with sulfuric acid, but the greater part is present in the form of salts of sodium and potassium.

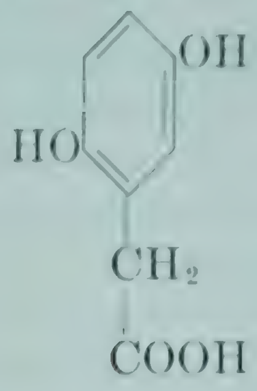
Levine[11] has shown that the urine of premature infants fed cow's milk regularly contains certain aromatic oxyacids, such as *p-hydroxyphenylpyruvic acid,* the product of the deamination of tyrosine. Administration of vitamin C abolishes this defect. A similar observation has been noted by Sealock in the case of scorbutic guinea pigs (see discussion, p. 1043).

[10a] Woodson, Hier, Solomon, and Bergeim: *J. Biol. Chem.*, **172**, 613 (1948).

[10b] Dent: *Biochem. J.*, **41**, 240 (1947); **43**, 169 (1948); *Biochem. Soc. Symposia*, **3** (*Partition Chromatography*), 34 (1950); Dent and Rose: *Quart. J. Med.*, **20**, 205 (1951).

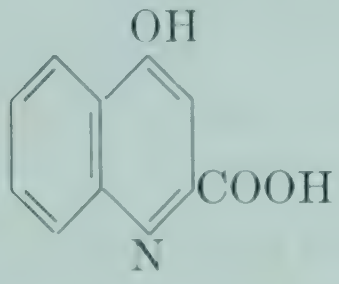
[11] Levine: *Science*, **90**, 620 (1939); *J. Clin. Invest.*, **22**, 551 (1943). See also Woolf and Edmunds: *Biochem. J.*, **47**, 630 (1950).

Homogentisic acid or 2,5-dihydroxyphenylacetic acid,



is another important oxyacid sometimes present in the urine. Under the name *glycosuric acid* it was first isolated from the urine by Marshall; subsequently Baumann isolated it and determined its chemical constitution. It occurs in cases of alcaptonuria (a so-called "inborn error of metabolism") and has also been found in the urine of the scorbutic guinea pig. A urine containing this oxyacid turns greenish-brown from the surface downward when treated with a little sodium hydroxide or ammonia. If the solution be stirred the color very soon becomes dark brown or even black. Homogentisic acid reduces alkaline copper solutions but not alkaline bismuth solutions.

Kynurenic acid or γ-hydroxy-β-quinoline carboxylic acid is a product of the metabolism of tryptophan but it appears to be excreted only after the ingestion of this amino acid in excess of normal requirements (see p. 1040).



Its presence after tryptophan administration in the urine of the dog, rat, rabbit, hyena, coyote, wolf, etc., and absence from that of the civet, cheetah, bear, raccoon, etc. (Jackson) may have significance in the zoological classification of species.

EXPERIMENT ON KYNURENIC ACID

Isolation of Kynurenic Acid. Acidify the urine with hydrochloric acid in the proportion 1:25. From this acid fluid both the uric acid and the kynurenic acid separate in the course of 24 to 48 hours. Filter off the combined crystalline deposit of the two acids, dissolve the kynurenic acid in dilute ammonia (uric acid is insoluble), and reprecipitate it with hydrochloric acid. If a solution containing kynurenic acid be evaporated to dryness with hydrochloric acid and potassium chlorate, a reddish residue is obtained which becomes first brownish green and then emerald green on adding ammonia (Jaffé).

Kynurenic acid may be quantitatively determined by Capaldi's method.[12]

PROTEIN

The nubecula of normal urine has been shown by one investigator to consist of a mucoid containing 12.7 per cent of nitrogen and 2.3 per cent of sulfur. This substance evidently originates in the urinary passages. It is probably slightly soluble in the urine. Some investigators believe that the material forming the nubecula of normal urine is nucleoprotein and not a mucin or mucoid. A discussion of nucleoprotein and related substances occurring in the urine under pathological conditions will be found in Chapter 29.

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[12] Capaldi: *Z. physiol. Chem.*, **23**, 92 (1897). Berg: *J. Biol. Chem.*, **91**, 513 (1931).

Normal urine contains a small amount of soluble protein—albumin, various enzymes (see below), etc.; the amount is too slight to be detected by any but the most delicate procedures. The significance of pathological proteinuria is discussed in Chapter 29.

CARBOHYDRATES

Normal human urine contains total reducing substances equivalent to about 0.05 to 0.15 per cent of glucose. Fermentable sugars make up about one-tenth of this or about 0.01 per cent. Whether all of the fermentable sugar is glucose is uncertain. Most of the sugar of normal urine is at any rate not glucose. The other sugars may include pentose, lactose, and altered carbohydrates formed by the baking of foods or through the action of bacteria in the intestinal tract. That glucose is not ordinarily excreted in more than traces is further indicated by the failure of glucose ingestion up to 1 g. per kg. of body weight to increase the fermentable urine sugar. In various types of nephritis the excretion of fermentable sugar is increased somewhat. Lactose may be found in the urine of pregnant women in appreciable amounts. In the rare conditions known as *pentosuria* and *fructosuria* these respective sugars may be present in readily detectable amount. Otherwise sugars are not normally found in sufficient amounts to give the ordinary sugar tests. Pathological glucosuria is discussed in Chapter 29.

ENZYMES

Various types of enzymes produced within the organism are excreted in both the feces and the urine. In this connection it is interesting to note that pepsin, trypsin, lipase, and an amylase have been positively identified in the urine. The amylase may be much increased in pancreatic disease.[13]

VITAMINS

The normal path of elimination of the fat-soluble vitamins is the intestinal tract. The water-soluble vitamins thiamine, riboflavin, ascorbic acid, and others, are excreted in the urine under normal conditions of vitamin nutrition, the amounts of these vitamins in the 24-hour excretion being directly proportional to the intake. Nicotinamide is normally eliminated in the form of the metabolite N^1-methylnicotinamide. The urinary output of the water-soluble vitamins under controlled conditions forms the basis of clinical tests for vitamin deficiencies, since in such states the tissues are unsaturated and tend to retain the test doses of vitamins.

In a study on seven healthy males on a normal dietary regime[14] the following ranges of values were found for daily vitamin intake and output:

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[13] Dodds: *Brit. J. Exptl. Path.*, **3**, 133 (1922).

[14] Denko, *et al.*: *Arch. Biochem.*, **10**, 33 (1946); **11**, 109 (1946).

Vitamin	Intake	Output		Average Excretion
		Urine	Feces	Per Cent
Thiamine, mg.	1.24– 1.63	0.144– 0.323	0.109–0.895	57
Riboflavin, mg.	1.74– 1.98	0.543– 0.913	0.823–1.313	91
Niacin, mg.	12.4 –20.9	1.13 – 1.39	2.14 –5.41	31
N^1-Methylnicotinamide, mg.	—	2.7 – 4.4	—	
Pantothenic acid, mg.	4.19– 5.30	2.68 – 3.46	0.89 –3.66	112
Pyridoxine, mg.	1.32– 2.46	0.57 – 0.69	0.33 –0.42	57
Folic acid, μg.	43–86	2.94 – 4.99	222–393	542
Biotin, μg.	37–54	27.8 –35.6	114–201	378
p-Aminobenzoic acid, μg.	97–220	131–198	183–361	230

* Per cent excretion = 100 (Urinary + Fecal output)/Intake.

The high excretion rates of the last three vitamins (particularly via the fecal route), is a reflection of the extent of synthesis by intestinal flora. The figures in this table do not represent the entire excretion of nicotinic acid and pyridoxine since their metabolites, N^1-methylnicotinamide and 4-pyridoxic acid, respectively, were not included.

VOLATILE FATTY ACIDS

Acetic, butyric, and formic acids have been found under normal conditions in the urine of man and of certain carnivora as well as in the urine of herbivora. Normally they arise principally from the fermentation of carbohydrates and the putrefaction of proteins. The acids containing the fewest carbon atoms (formic and acetic) are found to be present in larger percentage than those which contain a larger number of such atoms. The volatile fatty acids occur in normal urine in traces, the total output for 24 hours according to older investigators varying from 0.008 g. to 0.05 g. Formic acid excretion is increased in methyl alcohol poisoning.

LACTIC ACID

Lactic acid is supposed to pass into the urine when the supply of oxygen in the organism is diminished through any cause, e.g., in pneumonia, eclampsia, acute yellow atrophy of the liver, carbon monoxide poisoning, acute phosphorus poisoning, or epileptic attacks. This acid has also been found in the urine of healthy persons following the physical exercise incident to prolonged marching. Liljestrand and Wilson found the output of lactic acid to vary from 140 mg. to 1370 mg. after a few minutes of strenuous physical exercise e.g., stair running. (See experiment, Chapter 33.) Lactic acid has been detected in the urine of birds after the removal of the liver.

PHOSPHORYLATED COMPOUNDS

Phosphorus in organic combination has been found in the urine in such substances as glycerophosphoric acid, which may arise from the decomposition of lecithin, and phosphocarnic acid. It is claimed that on the average about 2.5 per cent of the total phosphorus elimination is in organic combination.

PIGMENTS

There are several pigments normally present in human urine, of which the most important is urochrome. Small amounts of urobilin, uroerythrin, and certain porphyrins (uroporphyrin and coproporphyrin) are also present.

Urochrome. Urochrome is the principal pigment of normal urine. Its chemical nature is not definitely established. It may be a compound of urobilin and urobilinogen with a peptide substance. It is a product of endogenous metabolism and is fairly constant in amount from day to day in the urine of normal individuals.

Urobilin. Urobilin is normally present in too small amount to give any appreciable color to the urine. It exists chiefly in the reduced form as the colorless urobilinogen which upon oxidation gives urobilin. (See Chapter 18.) It is derived from bile pigment by bacterial action in the bowel and most of it is excreted in the feces where the corresponding and probably identical compounds are known as stercobilinogen and stercobilin, respectively. Of the small amount absorbed the larger part is excreted in the bile.

PURINE BASES

The purine bases found in human urine are adenine, epiguanine (7-methylguanine), guanine, xanthine, heteroxanthine (7-methylxanthine), hypoxanthine, paraxanthine (1,7-dimethylxanthine), and 1-methylxanthine. The main bulk of the purine base content of the urine is made up of 7-methylxanthine, 1,7-dimethylxanthine, and 1-methylxanthine, which are derived for the most part from the caffeine, theobromine, and theophylline of the food. The total purine base content is made up of the products of two distinct forms of metabolism, i.e., metabolism of ingested nucleoproteins and purines and metabolism of tissue nuclear material. Purine bases resulting from the first form of metabolism are said to be of *exogenous* origin, whereas those resulting from the second form of metabolism are said to be of *endogenous* origin. The daily output of purine bases by the urine is extremely small and varies greatly with the individual (16 to 60 mg.). The output is increased after the ingestion of nuclear material as well as after the increased destruction of leukocytes. A well-marked increase accompanies leukemia. The output of purine bases by the urine is increased as a result of x-ray treatment. The purine bases form a higher percentage of the total purine excretion in the case of the monkey, sheep, and goat than in man.

EXPERIMENT ON PURINE BASES

Formation of the Silver Salts. **Add an excess of magnesia mixture[15] to 25 ml. of urine. Filter off the precipitate and add ammoniacal silver solution to the filtrate. A precipitate composed of the silver salts of the various purine bases is produced. The purine bases may be determined quantitatively by Kruger and Schmidt's method or Welker's method (see Chapter 31).**

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[15] See Appendix.

INORGANIC PHYSIOLOGICAL CONSTITUENTS

AMMONIA

Next to urea, ammonia is quantitatively the most important of the nitrogenous end products of protein metabolism. Ordinarily about 2.5 to 4.5 per cent of the total nitrogen of the urine is eliminated as ammonia, and on the average this would be about 0.7 g. per day. The significance of the ammonia content of the urine appears to be primarily if not entirely concerned with the mechanisms of acid-base balance in the body (see Chapter 24). If ammonia is fed in the form of oxidizable organic salts, such as ammonium acetate, ammonium lactate, etc., no extra ammonia appears in the urine. The organic portion of the salt is oxidized, and the ammonia portion is converted into urea and excreted in this form. If, however, a nonoxidizable salt such as ammonium chloride is administered, while the ammonia portion is probably also converted into urea for excretion (since there is no reason to suppose that the metabolism of the ammonium ion differs with different salts) the excretion of the extra chloride ions requires the simultaneous excretion of an equivalent amount of base. This base must be either sodium, potassium, or ammonium ions themselves, since these are the only forms of base available to the kidney. In the presence of abundant sodium or potassium (fixed base), the extra chloride is excreted largely as the sodium or potassium salt and no disturbance of acid-base balance results. If, however, the supply of fixed base is limited, its excretion results in the development of an acidosis due to the depletion of body base; in fact, the administration of a large dose of ammonium chloride is used clinically for the production of an acidosis. The kidney therefore *synthesizes* ammonia (volatile base) to the limit of its capacity under these conditions to conserve body base, the extra ammonia is excreted in the urine, and the urinary ammonia content rises. Excretion of acid phosphate also occurs here (see p. 818). That the ingested ammonia of the ammonium chloride plays no important part in the increased excretion of ammonia is shown by the fact that exactly the same condition occurs if hydrochloric acid is administered instead of ammonium chloride; there is a rise of urinary ammonia accompanying the increased excretion of chloride. Acid-forming foods (see Chapter 34) also increase the ammonia output, whereas the administration of alkalies or of base-forming foods decreases the excretion of ammonia. Copious water drinking increases the ammonia output. This fact has been interpreted as indicating a stimulation of the gastric secretion.

The acids formed during the process of protein destruction within the body (i.e., sulfuric acid formed by oxidation of the sulfur of methionine and cystine) have an influence upon the excretion of ammonia similar to that exerted by acids which have been administered. Therefore a pathological increase in the output of ammonia is observed in such diseases as are accompanied by an increased and imperfect protein metabolism. Likewise in diabetes mellitus, where the excretion of excessive amounts of acetoacetic acid and β-hydroxybutyric acid as their salts also tends to deprive the body of fixed base, increased excretion of ammonia occurs.

The kidney is the source of urinary ammonia. This is indicated by the fact that following extirpation of the kidneys the blood ammonia does not increase. On removal of the liver blood ammonia increases but not urinary ammonia. This indicates that the two have not the same origin. The blood ammonia appears to arise from the tissues, especially the muscles (probably from the deamination of adenylic acid) and to be converted so readily to urea that the blood ammonia remains too low to account for any appreciable part of the urinary ammonia. Ammonia is apparently formed by the cells of the tubules of the kidney. The precursor is believed to be an amide-nitrogen compound, probably *glutamine,* in blood plasma (Van Slyke) from which the enzyme *glutaminase* forms ammonia and glutamic acid. Deamination of amino acids by kidney tissue may also account for a small portion, possibly through the intermediate formation of glutamine. A decrease in the pH of the blood or of the kidney tissue seems to speed up the ammonia-producing mechanism.

The quantitative determination of ammonia must be made upon fresh or properly preserved urine, since upon standing normal urine will undergo ammoniacal fermentation (see p. 784).

EXPERIMENT ON AMMONIA

(See Exp. 2 under "Experiments on Phosphates," p. 819.)

SULFATES

Sulfur in combination is excreted in two forms in the urine: first, as unoxidized, loosely combined or neutral sulfur, and second, as oxidized or acid sulfur. The excretion of neutral sulfur has already been discussed (p. 807). The oxidized sulfur is eliminated chiefly in the form of the inorganic sulfate ion; a relatively small amount occurs in the form of ethereal sulfate, i.e., salts of sulfuric acid in combination with such aromatic substances as phenol, indole, skatole, cresol, pyrocatechol, and hydroquinol. This latter form of sulfuric acid is sometimes called conjugate sulfuric acid. The greater part of the total sulfur is eliminated in the oxidized form, but the absolute percentage of sulfur excreted in the various forms depends upon the total quantity of sulfur present; i.e., there is no definite ratio between the three forms of sulfur which will apply under all conditions. The preformed or inorganic sulfuric acid may be precipitated directly from acidified urine with $BaCl_2$, whereas the ethereal sulfuric acid must undergo a preliminary hydrolysis before it can be so precipitated.

The sulfuric acid excreted in the urine arises principally from the oxidation of the sulfur of protein within the body; a relatively small amount is due to ingested sulfates. Under normal conditions about 1.0 g. of total S is eliminated daily, about 75 to 95 per cent of this being in the form of sulfates. About 90 per cent of this sulfate excretion is in the form of inorganic sulfate and 10 per cent as ethereal sulfates and neutral sulfur.

The sulfate ion is excreted with greater difficulty than any other inorganic radical ordinarily present in normal blood. A retention of 30 times the normal blood value (0.9 to 1.1 mg. of S per 100 ml. of whole blood) has been observed. Sulfate ions are not readily absorbed by the tissues even when present in high concentration in the blood.

EXPERIMENTS ON SULFATES

1. *Detection of Inorganic Sulfuric Acid.* Place about 10 ml. of urine in a test tube, acidify with acetic acid, and add some barium chloride solution. A white precipitate of barium sulfate forms.

2. *Detection of Ethereal Sulfuric Acid.* Filter off the barium sulfate precipitate formed in the above experiment, add 1 ml. of hydrochloric acid and a little barium chloride solution to the filtrate and heat the mixture to boiling for 1 to 2 minutes. Note the appearance of a turbidity due to the presence of sulfuric acid which has been hydrolyzed from the ethereal sulfates and has reacted with $BaCl_2$ to form $BaSO_4$.

3. *Detection of Unoxidized or Neutral Sulfur.* Place about 10 ml. of urine in a test tube, introduce a small piece of zinc, add sufficient hydrochloric acid to cause a gentle evolution of hydrogen, and over the mouth of the tube place a filter paper saturated with lead acetate solution. In a short time the portion of the paper in contact with the vapors within the test tube becomes blackened due to the formation of lead sulfide. The nascent hydrogen has reacted with the loosely combined or neutral sulfur to form hydrogen sulfide, and this gas coming in contact with the lead acetate paper has caused the production of the black lead sulfide. Sulfur in the form of inorganic or ethereal sulfuric acid does not respond to this test. (For discussion of neutral sulfur compounds, see p. 807.)

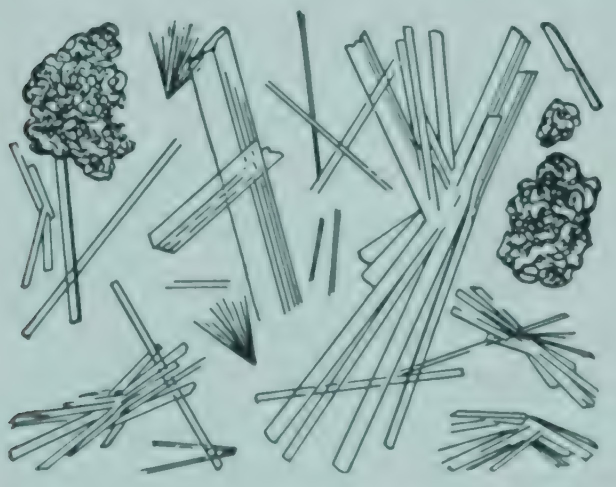

FIG. 225. CALCIUM SULFATE (HENSEL AND WEIL).

4. *Calcium Sulfate Crystals.* Place 10 ml. of urine in a test tube, add 10 drops of calcium chloride solution, and allow the tube to stand until crystals form. Examine the calcium sulfate crystals under the microscope and compare them with those shown in Fig. 225.

CHLORIDES

Next to urea, the various chlorides constitute the chief solid constituent of the urine. The excretion of chloride is dependent, in great part, upon the nature of the diet, but, on the average, the daily output is about 10 to 15 g. expressed as sodium chloride. Conditions which favor excessive perspiration, such as strenuous athletic or occupational activity, especially in a hot environment, cause a diminution in urinary output of chlorides, the chloride leaving the body by other channels. Muscular cramps may result when there is an excessive loss of sodium chloride from the body. To obviate this condition, workers in a high-temperature environment or persons indulging in very fatiguing muscular efforts, such as athletes in prolonged competition, make use of salt tablets. Because of their solubility, chlorides are never found in the urinary sediment.

The amount of chlorides excreted in the urine is related primarily to the chloride content of the food ingested. In cases of actual fasting the chloride content of the urine may be decreased to a slight trace which is derived from the body fluids and tissues. Under these conditions, however, an examination of the blood of the fasting subject will show the content of chloride in this fluid to be approximately normal. This forms a very striking example of the care nature takes to maintain the normal composition of the blood. There is a limit to the power of the body to maintain this equilibrium, however, and if the fasting organism be subjected to the influence of diuretics for a time, a point is reached where the normal composition of the blood can no longer be maintained and a gradual decrease in its chloride content occurs which finally results in death. Since the excreted chloride must carry base (e.g., sodium) along with it, death results not so much from the loss of chloride alone as from the inability of the organism to maintain a normal osmotic pressure in its body fluids, which is a major function of the sodium and chloride ions. Potassium cannot take the place of sodium in this respect, thus the administration of potassium chloride under these conditions is of no value whatever.

Pathologically, the excretion of chlorides may be decreased in some fevers, chronic nephritis, fasting, diarrhea, certain stomach disorders, and after extensive burns. Any condition accompanied by the formation of an exudate (e.g., pneumonia) will cause a diminished chloride output. In convalescence and with resolution of the exudate the chloride excretion rises again.

EXPERIMENTS ON CHLORIDES

Detection of Chlorides in Urine. **Place about 5 ml. of urine in a test tube, render it acid with nitric acid, and add a few drops of a solution of silver nitrate. A white precipitate, due to the formation of silver chloride, is produced. This precipitate is soluble in ammonium hydroxide.**

PHOSPHATES

Of the various inorganic anions of urine, phosphate is ordinarily second only to chloride in amount present. The excretion of phosphate is extremely variable, depending in large measure upon the diet, but on the average the total output for 24 hours is about 1.1 g. expressed as P. The bulk of this is in the form of inorganic phosphate, the organic phosphorus of the urine constituting only about 1 to 4 per cent of the total phosphorus content. The greater part of the inorganic phosphate arises from the ingested food, either from phosphate already present as such or more especially from the metabolism of compounds containing phosphorus in organic combination, such as *phosphoproteins, nucleoproteins* and *nucleotides*, and *phospholipides*. The various phosphorus-containing compounds of the body also contribute to the total output of this element. The phosphate content of the urine also depends to a considerable extent on conditions within the intestinal lumen; an increased alkalinity together with the presence of substances like calcium and magnesium which form insoluble phosphates tends to increase the proportion of phosphate excreted in the feces at the expense of the urinary phosphate content.

The phosphate ion is found in the urine in two forms, the acid phosphate or monobasic ion, $H_2PO_4^-$, and the dibasic ion, $HPO_4^=$. The ratio of these two ions determines in large measure the pH of the urine, since they constitute the major buffer system ordinarily present. In blood and in the glomerular ultrafiltrate of the kidney, these ions are present in a ratio corresponding to about 80 per cent basic phosphate and 20 per cent acid phosphate. If this ratio prevails in the urine, it will have the same pH as the blood, or approximately 7.4. Increased acidity of the urine is due to an increase in the amount of the acid phosphate relative to basic phosphate; at pH 6.6, which is approximately the pH of average urine, about 60 per cent of the total inorganic phosphate is in the acid form and 40 per cent appears as basic phosphate. The best available evidence indicates that the ability of the kidney to excrete a varying fraction of its total phosphate in either the acid or basic forms is an important part of the mechanism for the regulation of acid-base balance in the body; actually, the excretion of one equivalent of acid phosphate instead of basic phosphate corresponds to the excretion of one equivalent of hydrogen ion itself, and the simultaneous retention of one equivalent of base, as inspection of the formulas of these two salts will show. In combating acidosis, this mechanism appears to be of importance second only to the ability of the kidneys to replace sodium or potassium by ammonium. Available evidence indicates that the change from acid to basic phosphate and *vice versa* is brought about in the renal tubules, possibly by secretion of hydrogen ions (Pitts), since the total phosphate excretion does not vary with changes in the relative amounts of the acid and basic forms excreted.

If urine containing phosphate is rendered sufficiently alkaline, the so-called "alkaline earth" elements calcium and magnesium will precipitate, to the extent of their presence, as insoluble calcium and magnesium phosphates. This fraction of the total phosphate of urine was formerly called the "earthy phosphate" fraction as contrasted to the "alkaline phosphate" fraction composed of the soluble phosphates of sodium, potassium, and ammonium. It is doubtful whether this fractionation has any significance other than to indicate the amount of calcium and magnesium present relative to the total phosphate, and it should be abandoned.

The so-called "phosphaturia," the appearance of a copious crystalline precipitate which on examination proves to be magnesium ammonium phosphate ("triple phosphate"), ordinarily represents a decreased acidity of the urine and not an increased phosphate content. Such conditions may, however, be of significance in connection with a possible tendency to the formation of phosphate calculi. Measures designed to acidify the urine, and to decrease its phosphate content, have been of value in the treatment of this condition.

Pathologically the excretion of phosphoric acid is increased in such diseases of the bones as diffuse periostosis, osteomalacia, and rickets; according to some investigators, in the early stages of pulmonary tuberculosis, in acute yellow atrophy of the liver, in diseases which are accompanied by an extensive decomposition of nervous tissue, and after sleep induced by potassium bromide or chloral hydrate (Mendel). It is also increased after copious water drinking. A decrease in the excretion of phosphates is at times noted in febrile affections, such as the acute infectious diseases, in pregnancy, in the period during which the fetal bones are forming, and in diseases of the kidneys, because of nonelimination.

EXPERIMENTS ON PHOSPHATES

1. *Formation of "Triple Phosphate."* Place some urine in a beaker, render it slightly alkaline with ammonium hydroxide, add a small amount of magnesium sulfate solution, and allow the beaker to stand in a cool place overnight. Crystals of ammonium magnesium phosphate, "triple phosphate," form under these conditions. Examine the crystalline sediment under the microscope and compare the forms of the crystals with those shown in Fig. 226. If possible, examine the crystals from a freshly passed specimen of cloudy urine, or from a sample of urine which on standing for a short while after voiding has become cloudy. How do they compare with those obtained in this experiment?

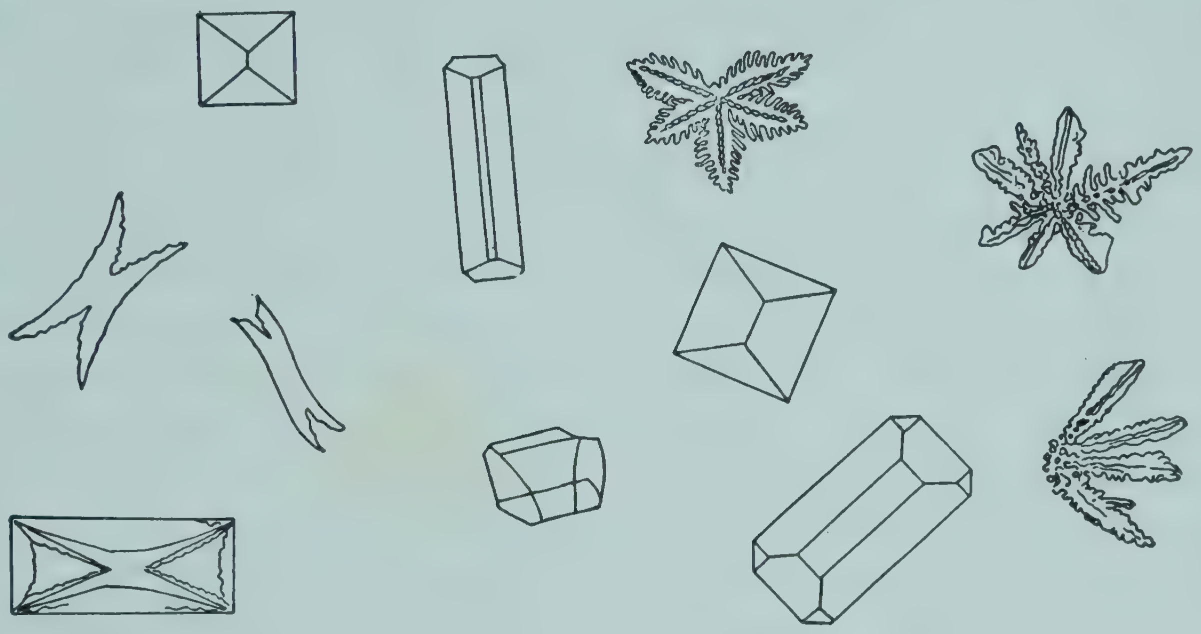

FIG. 226. TRIPLE PHOSPHATE (OGDEN).

2. *Ammoniacal Fermentation.* Stand some urine aside in a beaker for several days. Ammoniacal fermentation will develop and "triple phosphate" crystals will form.

***a.* Examine the sediment under the microscope and compare the crystals with those shown in Fig. 226.**

***b.* Hold a glass rod dipped in concentrated hydrochloric acid near the surface of the urine. Note the fumes of ammonium chloride.**

***c.* Insert a strip of red litmus paper in the urine. Permit the paper to dry. Note the gradual restoration of red color, due to volatilization of ammonia (volatile alkali). Run a control test using 0.5 per cent Na_2CO_3 (fixed alkali).**

SODIUM AND POTASSIUM

Sodium and potassium ions are always present in the urine. The amount of potassium excreted in 24 hours by an adult, subsisting upon a mixed diet, is on the average 1 to 3 g., whereas the amount of sodium under the same conditions is ordinarily 3 to 5 g. The ratio of K to Na is generally about 3:5. The absolute quantity of these elements excreted depends, of course, in large measure upon the nature of the diet. Because of the noningestion of NaCl and the accompanying destruction of potassium-containing body tissues, during fasting the urine contains more potassium than sodium.

Pathologically the output of potassium, in its relation to sodium, may be increased during fever; following the crisis, however, the output of this element may be decreased. It may also be increased in conditions associated with acidosis. In Addison's disease there is usually a relative retention of potassium and an increased excretion of sodium; the administration of cortical hormone preparations restores the normal relationship.

CALCIUM AND MAGNESIUM

The daily output of calcium in the urine, which depends principally upon the nature of the diet, is on the average about 0.1 to 0.3 g. per day. The percentage of calcium present in the urine at any one time (10 to 40 per cent of total calcium output) forms no dependable index as to the absorption of this element, since it may be again excreted into the intestine after absorption. Furthermore, as with phosphate (p. 817), the acidity or alkalinity of the intestinal lumen, and the presence of substances such as phosphate and fatty acids which form insoluble calcium salts, may determine to a considerable extent the relative output of calcium in the feces and the urine. Acidity promotes calcium absorption, alkalinity retards it. It is therefore impossible to draw any satisfactory conclusions regarding the excretion of calcium unless accurate analytical data from both the feces and the urine are obtained.

Myers and Fine[16] have reported data showing a comparison of the kidney and intestine as excretory routes for various inorganic constituents. Their findings in this connection are summarized in the following table:

Number of Cases	*Moisture Content of Feces (Per Cent)*	*Fecal Output in Per Cent of Total in Urine and Feces*							
		H_2O	N	S	Cl	P	Ca	Mg	K
5	76	6	10	10	3	36	90	72	18
9	84	16	15	19	9	33	89	68	27

The average findings in five cases with well-formed stools, 74 to 79 per cent moisture, and those with diarrheal stools, 79 to 89 per cent moisture, have been grouped separately in the table. It is not believed that the findings differed especially from the normal, except in that group of cases which suffered from intestinal diarrhea.

Very little is known positively regarding the actual course of the excretion of calcium under pathological conditions. An excess is found in some diseases of the bones, e.g., osteomalacia. In others, as in rickets, the urinary excretion may be very low.

The daily excretion of magnesium by way of the urine usually amounts to between 0.05 and 0.2 g. The amount depends mainly on the diet. About 65 per cent or more of the excreted magnesium is usually eliminated by the feces; the remainder passes out in the kidneys. There may be a retention of magnesium in certain bone disorders accompanying a loss of calcium, for example, in osteomalacia. Thus the excretion of calcium and magnesium do not necessarily run parallel.

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[16] Myers and Fine: *Proc. Soc. Exptl. Biol. Med.*, **16**, 73 (1919).

EXPERIMENT ON CALCIUM

Sulkowitch Test. This is a qualitative test for calcium in the urine. It has applications in parathyroid derangement, urinary calculi, infantile tetany, severe nephritis, etc.

Procedure. **Place the patient on a diet containing sufficient calcium for his needs, and collect a 24-hour specimen of urine. To 5 ml. of the mixed urine sample add 2 ml. of the Sulkowitch reagent[17] dropwise. If no precipitate forms immediately, mix the contents of the tube thoroughly and allow to stand.**

INTERPRETATION. In case there is *no precipitate* there is of course no calcium in the urine. From this finding it is concluded that the blood serum probably contains not more than 5 to 7.5 mg. per 100 ml. If the precipitate forms as a fine white cloud, the calcium content of the serum is considered to fall within the *normal range* of 9 to 11 mg. per 100 ml. However if a heavy, milklike precipitate forms, there is danger of *hypercalcemia.* In routine examinations, the precipitates may be graded 1, 2, 3 or 4.

CARBONATES

Carbonate, in the form of the bicarbonate ion, HCO_3^-, generally occurs in small amount in the neutral or alkaline urine of man and carnivora, whereas much larger quantities are ordinarily present in the consistently alkaline urine of herbivora. The alkaline reaction of the urine of herbivora is due in great measure to the presence of bicarbonate. The carbonates of the alkaline earths are sometimes found in amorphous urinary sediments from quite alkaline urine.

IRON

Iron is present in small amount in normal urine. It probably occurs partly in inorganic and partly in organic combination. The iron contained in urinary pigments or chromogens is in organic combination. According to different investigators the iron content of normal urine probably averages not more than 1 to 2 mg. per day. Mitchell and Hamilton[18] report that sweat contains 1–2 mg. of iron per liter. After splenectomy there is an increased loss of iron from the body particularly by way of the feces.

EXPERIMENT ON IRON

Detection of Iron in Urine. **Evaporate a convenient volume (10 to 15 ml.) of urine to dryness. Incinerate and dissolve the residue in a few drops of iron-free hydrochloric acid and dilute the acid solution with 5 ml. of water. Divide the acid solution into two parts and make the following tests: (*a*) To the first part add a solution of ammonium thiocyanate; a red color indicates**

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[17] 2.5 g. of oxalic acid, 2.5 g. of ammonium oxalate and 5 ml. of glacial acetic acid, made up to 150 ml. with distilled water.

[18] Mitchell and Hamilton: *J. Biol. Chem.*, **178**, 345 (1950).

the presence of iron. (*b*) To the second part of the solution add a little potassium ferrocyanide solution; a precipitate of prussian blue forms upon standing.

FLUORIDES, NITRATES, AND SILICATES

These substances are all found in traces in human urine under normal conditions. Nitrates are undoubtedly introduced into the organism in the water and ingested food. The average excretion of nitrates is about 0.5 g. per day, the output being the largest upon a vegetable diet and smallest upon a meat diet. Nitrites are found only in urine which is undergoing decomposition and are formed from nitrates in the course of ammoniacal fermentation.

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29

Urine: Pathological Constituents

Many of the substances considered in this chapter as pathological constituents of urine are present in small amount in normal urine. Hence their pathological significance may be more a question of the amount present than of their actual presence in or absence from urine. It is generally true, however, that the usual qualitative tests for these substances are of such sensitivity as to yield an essentially negative result when applied to normal urine, but to respond readily when unusual amounts are present. In this connection it is well to remember that a single specimen of urine may be sufficiently concentrated or otherwise influenced by dietary or other factors as to yield a positive result with a particular test, the significance of which disappears, however, when considered in the light of the total 24-hour excretion. Whenever possible, therefore, unless it is desired otherwise for special purposes, it is recommended that urine tests be carried out on a portion of the well-mixed and properly preserved 24-hour urine before interpreting results; if this is not feasible, the overnight sample collected before breakfast should be used.

GLUCOSE

Traces of this sugar may occur in normal urine, but the amount is ordinarily too small to be demonstrable by the common qualitative tests (see also Chapter 31). The presence of readily detectable amounts of glucose in urine is known as *glucosuria;* the term *glycosuria* is frequently used, but this expression more correctly refers to the presence of any sugar, not necessarily glucose, in the urine. Glucosuria may be either benign or pathological, and it is important to distinguish between these two types. Renal diabetes, in which the kidney threshold for glucose is below normal but the blood sugar level is normal, is an example of a benign glucosuria, as are the glucosurias associated with alimentary and emotional hyperglycemias. Pathological glucosurias include chiefly those of diabetes mellitus and other endocrine disorders, in which there is a marked elevation of the blood sugar and usually an increased volume of urine (polyuria). The glucose content of the urine in diabetes mellitus may be as high as 10 per cent or more, but is usually around 3 to 5 per cent. The urine may be light in color and have a high specific gravity.

TESTS FOR GLUCOSE

The various tests for glucose in the urine which are embraced in the experiments given herewith are based upon one of the following properties of this sugar, as discussed in Chapter 2: (1) Its power to *reduce the ions of certain metals in alkaline solution;* (2) its power to *rotate the plane of polarized light;* (3) its power to *form a crystalline osazone with phenylhydrazine;* and (4) its ability to *ferment with ordinary yeast.*

None of these tests by itself is specific for glucose. Positive evidence that the sugar is glucose may be obtained by demonstrating that the reducing power disappears after yeast fermentation and that a typical glucosazone is obtained in the absence of a positive test for fructose or mannose (see below). Quantitative measurement of optical rotation in relation to total reducing power is also of value.

The official test for glucose adopted by the Association of Life Insurance Medical Directors of America may be found in Chapter 31.

1. Phenylhydrazine Reaction. Yellow crystalline compounds called *osazones* are formed from certain sugars by reaction with phenylhydrazine, in general each individual sugar giving rise to an osazone of a definite crystalline form which is typical for that sugar.

As applied to urine, however, it is frequently difficult to obtain characteristic crystals, even for glucose, since crystal form and growth may be influenced by other substances present. Better results are usually obtained if the urine is clarified first, as described below. Results are more significant if they are positive (i.e., if characteristic crystals are obtained) than if they are negative, but in any event the test should be regarded as a guide or confirmation, to be accompanied by more specific tests. Identification of the individual sugar osazones by their melting points is of little value.

In this connection it is important to remember that of the simple sugars of interest in physiological chemistry, glucose, fructose, and mannose give the same osazone. Fructose may be ruled out by the absence of a positive Selivanoff test (see p. 848); the presence of mannose will be indicated by the formation of a colorless crystalline *hydrazone* on treatment with phenylhydrazine in the cold, prior to the heating which produces the osazone.

Procedure. **To a small amount of phenylhydrazine mixture[1] (about one-half inch in a small test tube), add 5 ml. of the urine, shake well, and heat on a boiling water bath for one-half to three-quarters of an hour. Allow the tube to cool slowly (not under the tap) and examine the crystals microscopically (see Plate II, opposite p. 63). If the solution has become too concentrated in the boiling process it will be light red in color and no crystals will separate until it is diluted with water.**

In case doubtful results are obtained by this test owing to the presence of interfering substances, the urine should be clarified and the test repeated. To clarify the urine introduce 10 ml. into a test tube, add 1 g. of pure blood charcoal, heat to boiling and allow to stand with occasional shaking for five minutes, then filter. Use the filtrate in the test.

2. Reduction Tests. It is to their potential aldehyde or ketone structure that many sugars owe the property of readily reducing alkaline solutions of metals like copper, bismuth, mercury, and iron; they also possess the property of reducing ammoniacal silver solutions with the separation of metallic silver. Upon this property of reduction the most widely used tests for sugars are based. A positive reduction test is not specific for glucose, or even for reducing sugars in general; non-sugar-reducing material is present in traces in normal urine and may be sufficiently augmented in amount, particularly in concentrated urines, to give a positive test. Other factors which may influence interpretation of results are discussed below. The chemistry of the various reduction tests and reagents is discussed in Chapter 2.

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[1] See Appendix.

a. Fehling's Test. **To about 1 ml. of Fehling's solution[2] in a test tube add about 4 ml. of water, and boil.[3] This is done to determine whether the solution will of itself cause the formation of a precipitate of brownish-red cuprous oxide. If such a precipitate forms, the Fehling's solution must not be used. Add urine[4] to the hot Fehling's solution, a few drops at a time, and heat the mixture to boiling after each addition (never add more urine than the original volume of Fehling's solution). The production of yellow or brownish-red cuprous oxide indicates that reduction has taken place. The yellow precipitate is more likely to occur if the urine is added rapidly and in large amount, whereas with a less rapid addition of smaller amounts of urine the brownish-red precipitate is generally formed. The differences in color of the cuprous oxide precipitates under different conditions are apparently due to differences in the size of the particles, the more finely divided precipitates having a yellow color, while the coarser ones are red. In the presence of protective colloidal substances the yellow precipitate is usually formed.**

This classical test is not entirely reliable when used to detect sugar in the urine, and has been largely replaced by Benedict's test (see below). Such compounds as conjugate glucuronates, uric acid, nucleoprotein, and homogentisic acid, when present in sufficient amount, may produce a result similar to that produced by sugar. Phosphates of the alkaline earths may be precipitated by the alkali of the Fehling's solution as a grayish-white precipitate which should not be mistaken for the cuprous oxide. Cupric hydroxide may also be reduced to cuprous oxide and this in turn may form a soluble complex with creatinine, a normal urinary constituent. This will give the urine under examination a greenish tinge and may obscure the sugar reaction even when a considerable amount of sugar is present.

Conjugate glucuronates are present in normal urine in small amount (see p. 842) and are increased in amount after the ingestion of such substances as chloral hydrate, camphor, menthol, thymol, antipyrine, phenol, etc. The chloral hydrate is excreted in the urine as trichloroethylglucuronate. This compound reduces Fehling's solution and is levorotatory, whereas glucose also reduces but is dextrorotatory. Therefore by means of a polariscopic test a "chloral urine" may be differentiated from a "sugar urine."

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[2] See Appendix.

[3] More dilute Fehling's solution should be used in testing urines containing small amounts of sugar. In case of urines containing a high concentration of sugar it may sometimes be desirable to use a larger volume of Fehling's solution.

[4] In case doubtful results are obtained by this test owing to the presence of interfering substances the urine should be clarified with charcoal as described above, and the test repeated.

In testing urine preserved by chloroform a positive test may be obtained in the absence of sugar. This is due to the fact that the hot alkali produces a reducing substance from the chloroform.

Ammonium salts also interfere with Fehling's test preventing the precipitation of cuprous oxide. If they are present in excess, the urine should be made alkaline with strong sodium carbonate, and boiled (or, better, aerated with a vigorous stream of air) to decompose and liberate the ammonia.

b. Benedict's Test. **This is the most satisfactory of the copper reduction tests, and in laboratory practice has largely replaced Fehling's. The following is the procedure for the detection of glucose in urine: To 5 ml. of the reagent[5] in a test tube add exactly 8 drops of the urine to be examined. The fluid is then boiled vigorously for from one to two minutes and then allowed to cool spontaneously. (Do not hasten cooling by immersion in cold water.) The test may also be carried out by heating for five minutes in a boiling water bath, removing, and allowing to cool in the air. This procedure is recommended for serial tests. If a water bath is used, it is important that the urine and reagent be thoroughly mixed before placing in the water bath. In the presence of glucose the entire body of the solution will be filled with a colloidal precipitate, which may be green, yellow, or red in color, depending upon the amount of sugar present. In the presence of over 0.2 to 0.3 per cent of glucose, the precipitate will form quickly. If no glucose is present, the solution will either remain perfectly clear, or will show a very faint turbidity, due to precipitated urates.**

Even very small quantities of glucose in urine (0.1 per cent) yield precipitates of surprising bulk with this reagent, and the positive reaction for glucose is the filling of the entire body of the solution with a precipitate, so that the solution becomes opaque. Since amount rather than color of the precipitate is made the basis of this test, it may be applied, even for the detection of small quantities of glucose, as readily in artificial light as in daylight. Chloroform does not interfere with this test nor does uric acid or creatinine interfere to such an extent as in the case of Fehling's test. It is common clinical practice to run this test under consistently uniform conditions (same amount of urine, heating time, etc.) and to indicate the intensity of the reaction by arbitrary signs (+, ++, +++, etc.) as an index not only of the presence of sugar but also of its approximate relative concentration.[6]

c. Bismuth Reduction Test (Nylander). **To 5 ml. of urine in a test tube add one-tenth its volume of Nylander's reagent[5] and heat for 5 minutes in a**

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[5] See Appendix.

[6] A simple modification of the copper reduction test for urine sugar is available commercially in the form of tablets containing copper sulfate, sodium hydroxide, and citric acid ("Clinitest" tablets, obtainable from the Ames Co., Inc., Elkhart, Indiana). In use, a little urine is diluted with 2 volumes of water and a tablet added to the mixture. Application of external heat is unnecessary. After the mixture has stood for a few moments, the typical appearance of a positive copper reduction test is obtained if the urine contains reducing sugar.

boiling water bath.[7] The mixture will darken if reducing sugar is present, and upon standing for a few moments a black color will appear.

This color is due to the precipitation of bismuth.[8] If the test is made on urine containing albumin this must be removed, by boiling and filtering, before applying the test, since with albumin a similar change of color is produced. Glucose when present to the extent of 0.08 per cent or even less may be easily detected by this reaction. Uric acid, creatinine, and homogentisic acid which interfere with the Fehling test, do not interfere with the Nylander reaction.

Urine rich in indican, uroerythrin, urochrome, or porphyrins, as well as urine excreted after the ingestion of large amounts of certain drugs, may give a darkening of the Nylander's reagent similar to that of a true sugar reaction. It has been claimed that the urine after the administration of urotropin will reduce the Nylander reagent.

A positive bismuth reduction test is probably due to the following reactions:

$$Bi(OH)_2NO_3 + KOH \rightarrow Bi(OH)_3 + KNO_3$$
$$2Bi(OH)_3 - 3O \rightarrow 2Bi + 3H_2O$$

Before testing the urine, Bohmansson treats 10 ml. with 2 ml. of 25 per cent hydrochloric acid and 4 to 5 g. of boneblack. This mixture is shaken one minute, then filtered, and the neutralized filtrate tested by Nylander's reaction. He claims that this procedure removes certain interfering substances, notably urochrome.

3. Fermentation Tests.

a. Saccharometer Method. **Rub up in a mortar about 15 ml. of urine with a small piece of compressed yeast or with about 0.5 g. active dry yeast. Treat in the same way 15 ml. of urine known not to contain glucose and 15 ml. of urine to which glucose has been added. These are necessary controls. Transfer each mixture to a saccharometer (Fig. 15, p. 69) and stand it aside in a warm place for about 12 hours. If glucose is present, alcoholic fermentation will occur and carbon dioxide will collect as a gas in the upper portion of the tube. On the completion of fermentation, fill the bulb portion of the saccharometer with 10 per cent sodium hydroxide solution, close the opening of the apparatus tightly with the thumb, mix the contents thoroughly, and restore the gas space to the limb of the saccharometer. Holding the apparatus at arm's length and with face averted (to avoid accidents) remove the thumb from the opening. Remembering that sodium hydroxide has the power to absorb carbon dioxide, how do you explain the result?**

Ordinary bakers' yeast (*Saccharomyces cerevisiae*) will readily ferment glucose, fructose, mannose, maltose, and sucrose. Galactose, lactose, and the various pentoses are not fermentable, or, if so, at such a slow rate as to be of no practical significance in connection with the above test; yeast which readily ferments galactose may be obtained, however, by incubation and growth of ordinary baker's yeast on a medium rich in galactose. Thus the yeast fermentation test is of considerable practical value in distinguishing between glucosuria, lactosuria, and pentosuria, either simple or mixed, particularly when exhaustive yeast fermentation is used in connection with reduction tests before and after treatment with yeast, as in Mathews' procedure which follows.

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[7] Hammarsten suggests that the solution be boiled for two to five minutes (according to the sugar content) over a free flame and the tube then permitted to stand five minutes before drawing conclusions.

[8] A dry powder containing the ingredients for a Nylander test is available commercially under the name of "Galatest" powder (obtainable from the Denver Chemical Mfg. Co., Inc., New York City). In use, a drop of urine is placed on a small amount of the powder. If reducing sugar is present, the powder turns gray or black.

b. *Mathews' Modification for Distinguishing Fermentable Sugar (Glucose) from Nonfermentable Reducing Substances (Lactose, etc.).* Mix 20 ml. of urine in a test tube with 2–3 g. of active dry yeast, stirring vigorously. Place at an angle of about 45° in a beaker of water and keep at 42° C. for 50 minutes, mixing occasionally. If 1 per cent or more of glucose is present, an evolution of gas bubbles will be observed after a few minutes and all glucose will be decomposed (up to 6 per cent) in 50 minutes. Filter and apply Benedict's test. A positive test indicates the possibility of lactose, but only if a control test using the same amount of yeast with urine containing added glucose demonstrates that the yeast is capable of removing all glucose under the experimental conditions. Reducing sugar may also be determined quantitatively before and after the fermentation.

4. Polariscopic Examination. For directions as to the use of the polariscope, see p. 69, and for application to the quantitative analysis of urine, Chapter 31.

PROTEINS

Normal urine contains a trace of protein material, but the amount present is so slight as to escape detection by any of the simple tests in general use for the detection of protein urinary constituents. The following are the more important forms of protein material which have been detected in the urine under pathological conditions:

1. Serum albumin
2. Serum globulin
3. Non-blood proteins
    - Proteoses and peptones
    - Proteins of Bence-Jones and of Hektoen, Kretschmer, and Welker
4. Nucleoprotein
5. Fibrin
6. Oxyhemoglobin and related compounds
7. Myoglobin

ALBUMIN

Normal urine contains a trace of albumin which is too slight to be detected by the usual procedures. *Albuminuria* is the condition in which readily detectable amounts of the serum proteins are found in the urine. The proteinuria is most commonly due to the presence of serum albumin, since albumin is the most abundant of these serum proteins, and has the smallest molecular size, thus permitting the greatest diffusion through damaged membranes. It is not uncommon, however, to find significant amounts of globulin material in so-called albuminuria if the proper tests are applied. There is some uncertainty as to whether or not the serum proteins are altered from their normal state when found in the urine.

There are two distinct forms of albuminuria, viz., renal albuminuria and accidental albuminuria. Sometimes the terms *true albuminuria* and *false albuminuria* are substituted for those just given. In the renal type the albumin is excreted by the kidneys. This indicates a more serious condition and at the same time is more frequently encountered than the accidental type. Associated with renal albuminuria are usually altered blood pressure or kidney structure. In the accidental form of albuminuria the albumin is *not* excreted by the kidneys (as is the case in the renal form of the disorder) but arises from the blood, lymph, or some albumin-containing exudate coming into contact with the urine at some point below the kidneys.

The determination of albumin may be of assistance in following the course of kidney disturbances, but the results can only be interpreted in the light of other clinical findings. Even in nephritis not every sample of urine will be abnormal if the specimens are obtained under ordinary conditions. It is nevertheless rare in this condition to fail to find albumin, casts, or excessive red cells and white cells in a concentrated urine. No diagnosis of the presence or absence of nephritis should be made until such findings are observed.

Benign proteinuria, which is usually transitory and in which there is no evidence of permanent kidney damage, may be due to such causes as severe exercise or cold baths. It is particularly common in young people. In the unusual condition known as *orthostatic albuminuria*, the proteinuria is apparently due to posture; urine formed while the patient is lying down is free from protein, while that formed when the upright position is assumed, particularly the position of military attention, contains protein. This condition is apparently harmless, although it has been used as a deterrent to military service, and may be due, in some instances at least, to mechanical pressure on the renal blood vessels due to a lumbar lordosis.

Foreign proteins injected into the blood stream are excreted by way of the urine, and lead to some excretion of blood proteins also. Pathological albuminuria may in some cases be due to the passage of an abnormal protein from the tissues into the blood.

TESTS FOR ALBUMIN

The urine should be filtered or centrifuged before these tests are performed, and care should be taken against misinterpretation of the presence of pus or bacteria. None of the tests described here is specific for albumin, since practically all of the proteins which have been found in urine at one time or another will respond to most of these tests. It is common clinical practice, however, to refer to a positive reaction as signifying the presence of albumin in the absence of specific information that some other protein (globulin, hemoglobin, Bence-Jones protein, etc.) is present, since in the vast majority of cases it appears to be really albumin which is concerned. A positive test for albumin would entail its characterization as a heat-coagulable protein, soluble in salt-free water (i.e., after dialysis), and requiring more than half-saturated ammonium sulfate for precipitation (see Chapter 6 for further discussion).

The official test for albumin adopted by the Association of Life Insurance Medical Directors of America may be found in Chapter 31.

1. *Nitric Acid Ring Test* (*Heller*). Place 5 ml. of concentrated HNO_3 in a test tube, incline the tube, and by means of a pipet allow the urine to flow slowly down the side. The liquids should stratify with the formation of a white zone of precipitated protein at the zone of contact.

If the albumin is present in very small amount the white zone may not form until the tube has been allowed to stand for several minutes. If the urine is quite concentrated, a white zone, due to uric acid or urates, will form upon treatment with nitric acid as indicated. This ring may be easily differentiated from the albumin ring by repeating the test after diluting the urine with 3 or 4 volumes of water, whereupon the ring, if due to uric acid or urates, will not appear. It is ordinarily possible to differentiate between the albumin ring and the uric acid ring without diluting the urine, since the ring, when due to uric acid, has ordinarily a less sharply defined upper border, is generally broader than the albumin ring, and frequently is situated in the urine above the point of contact with the nitric acid. Concentrated urines also occasionally exhibit the formation, at the point of contact, of a crystalline ring with very sharply defined borders. This is urea nitrate and is easily distinguished from the fluffy ring of albumin. If there is any difficulty in differentiation, a simple dilution of the urine with water, as above described, will remove the difficulty. Various colored zones, due either to the presence of indican, bile pigments, or to the oxidation of other organic urinary constituents, may form in this test under certain conditions. These colored rings should never be confounded with the *white* ring which alone denotes the presence of albumin.

After the administration of certain drugs, a white precipitate of resin acids may form at the point of contact of the two fluids and may cause the observer to draw wrong conclusions. This ring, if composed of resin acids, will dissolve in alcohol, whereas the albumin ring will not dissolve in this solvent.

A ring closely resembling the albumin ring is often obtained in urines preserved for a considerable time by thymol when subjected to the nitric acid test. The ring is due to the formation of nitrosothymol and possibly nitrothymol. If the thymol is removed from the urine by extraction with petroleum ether[9] previous to adding nitric acid, the ring does not form.

2. *Nitric Acid and Magnesium Sulfate Ring Test* (*Roberts*). Place 5 ml. of Roberts' reagent[10] in a test tube, incline the tube, and by means of a pipet allow the urine to flow slowly down the side. The liquids should stratify with the formation of a white zone of precipitated protein between the layers.

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[9] Accomplished readily by gently agitating equal volumes of petroleum ether and the urine under examination for *two minutes* in a test tube before applying the test.

[10] See Appendix.

This test is a modification of Heller's ring test and is rather more satisfactory than that test, since the colored rings never form and consequent confusion is avoided.

3. *Sulfosalicylic Acid Test.* To 1 volume of urine add 2 to 3 volumes of 3 per cent sulfosalicylic acid solution. A turbidity (compare against a control diluted with water) or precipitate denotes the presence of albumin or globulin (but not proteose). The precipitate may be intensified by warming.

4. *Coagulation or Boiling Test. a.* Heat 5 ml. of urine to boiling in a test tube. (If the urine is not clear it should be filtered.) A precipitate forming at this point is due either to albumin (or globulin) or to phosphates. Acidify the urine slightly by the addition of 3 to 5 drops of very dilute acetic acid, adding the acid drop by drop to the hot solution. If the precipitate is due to phosphates it will disappear under these conditions, whereas if it is due to protein it will not only fail to disappear but will become more flocculent in character, since the reaction of a fluid must be acid to secure the complete precipitation of the protein by this coagulation process.

Too much acid should be avoided since it will cause the protein to go into solution. Certain *resin acids* may be precipitated by the acid, but the precipitate due to this cause may be easily differentiated from the protein precipitate by reason of its solubility in alcohol.

***b.* A modification of this test in quite general use is as follows: Fill a test tube two-thirds full of urine and gently heat the upper half of the fluid to boiling, being careful that this fluid does not mix with the lower half, which serves as a control. A turbidity indicates protein or phosphates. Acidify the urine slightly by the addition of 3 to 5 drops of dilute acetic acid, whereupon the turbidity, if due to phosphates, will disappear.**

5. *Osgood-Haskins Test for Urinary Protein.* To 5 volumes of urine add 1 volume of 50 per cent acetic acid, followed by 3 volumes of saturated (30 per cent) sodium chloride. (The appearance of a precipitate after the addition of acetic acid, at room temperature, indicates the presence of bile salts, urates, resin acids, etc., whereas a precipitate after the addition of the salt solution suggests Bence-Jones protein (see p. 832), or globulin in excess of 0.38 g. per liter.) Heat the mixture gradually to boiling. As the temperature is raised the precipitate of Bence-Jones protein, if present, will go into solution; if albumin or globulin are present a precipitate will form. This test has the advantage of indicating the presence of Bence-Jones protein as well as albumin and globulin.

GLOBULIN

Serum globulin is not a constituent of normal urine but frequently occurs in the urine under pathological conditions and is ordinarily associated with serum albumin. In albuminuria globulin in varying amounts often accompanies the albumin, and the clinical significance of the two is very similar. Under certain conditions globulin may occur in the urine unaccompanied by albumin.

TESTS FOR GLOBULIN

Globulin will respond to all the tests outlined above under "Albumin." If it is desirable to differentiate between albumin and globulin in any urine, the following procedure may be employed:

Place 25 ml. of neutral urine in a small beaker and add an equal volume of a saturated solution of ammonium sulfate. Globulin, if present, will be precipitated. If no precipitate forms, add ammonium sulfate *in substance* to the point of saturation. If albumin is present it will be precipitated upon saturation of the solution as just indicated. This method may also be used to separate globulin and albumin when they occur in the same urine.

Frequently in urine which contains a large amount of urates a precipitate of ammonium urate may occur when the ammonium sulfate solution is added to the urine. This urate precipitate should not be confounded with the precipitate due to globulin. The two precipitates may be differentiated by means of the fact that the urate precipitate ordinarily appears only after the lapse of several minutes whereas the globulin generally precipitates at once.

NONBLOOD PROTEINS

Certain proteins are occasionally excreted by the kidneys which do not give the precipitin reactions for any of the normal blood proteins. These include proteoses, Bence-Jones protein, and the protein of Hektoen, Kretschmer, and Welker.

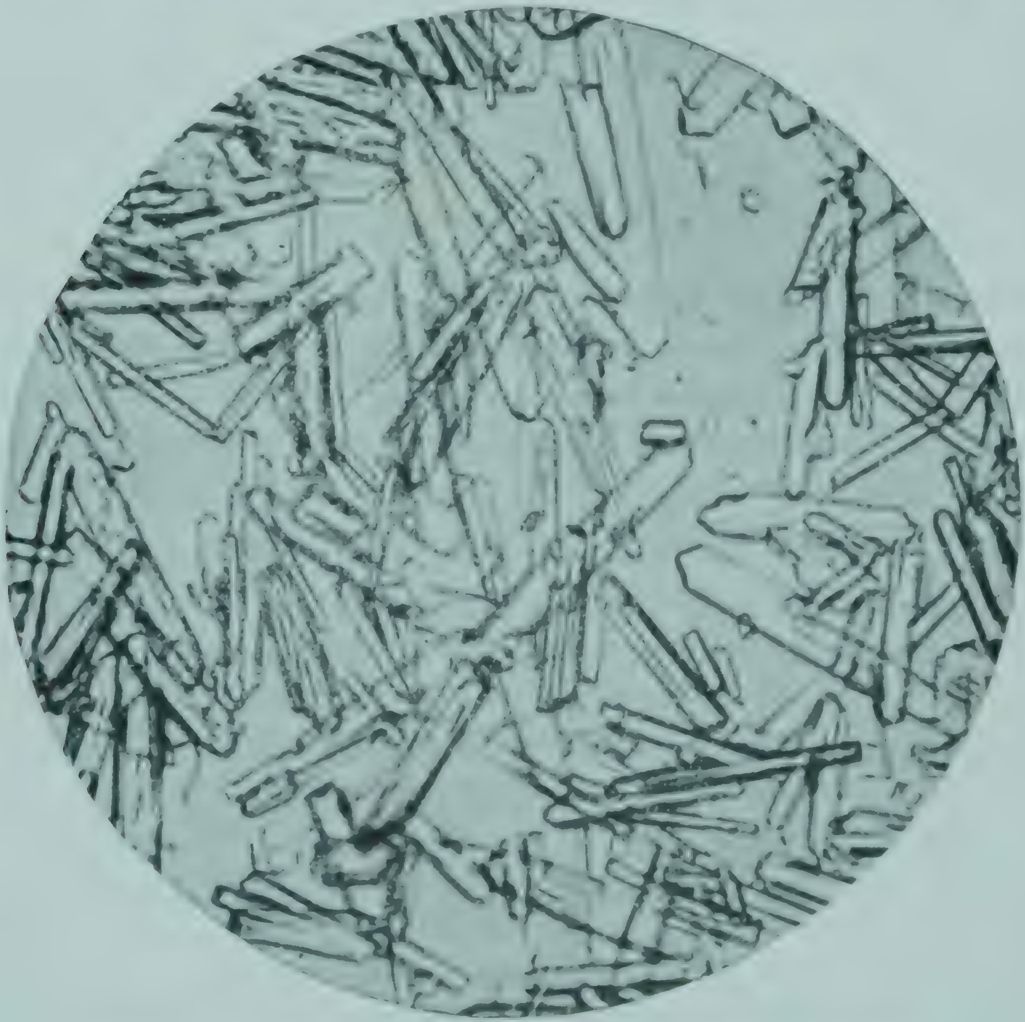

FIG. 227. CRYSTALLINE BENCE-JONES PROTEIN.
Isolated by Summerson from the urine of a case of multiple myeloma.

Proteoses or substances giving similar precipitation reactions have frequently been found in the urine in cases of pneumonia, diphtheria, intestinal ulcer, carcinoma, dermatitis, osteomalacia, atrophy of the kidneys, and in conditions in which there is absorption of partially digested pus.

Bence-Jones protein is believed to be of diagnostic importance in cases of multiple myeloma and myelogenic osteosarcoma. It has been shown to be chemically and immunologically distinct from any of the blood proteins and has been obtained in crystalline form (see Fig. 227). It appears to be present normally in small amount in the bone marrow and in certain white blood cells. Its excretion in relatively large amount (30 to 50 g. per day in some instances) under pathological conditions is apparently due either to overproduction or decreased utilization; it is not known which is the true explanation. The presence of Bence-Jones protein in the blood plasma of patients with multiple myeloma has been shown by electrophoretic methods.

The protein of Hektoen, Kretschmer, and Welker resembles the Bence-Jones protein and certain proteoses in solubility and precipitation limits with ammonium sulfate (40 to 60 per cent). It is distinct from these, and from blood proteins in its precipitin reactions, its behavior with heat, and in crystalline form.

TEST FOR NONBLOOD PROTEINS

1. *Schulte's Method.* Acidify 50 ml. of urine with dilute acetic acid and filter off any precipitate of nucleoprotein which may form. Now test a few ml. of the urine for coagulable protein, by test 4 under "Albumin," p. 831. If coagulable protein is present, remove it by coagulation and filtration before proceeding. Introduce 25 ml. of the urine, freed from coagulable protein, into 150 ml. of absolute alcohol and allow it to stand for 12 to 24 hours. Decant the supernatant fluid and dissolve the precipitate in a small amount of hot water. Now filter this solution, and after testing again for nucleoprotein with *very dilute* acetic acid, try the biuret test. If this test is positive the presence of proteose is indicated.[11]

Urobilin does not ordinarily interfere with this test since it is almost entirely dissolved by the absolute alcohol when the proteose is precipitated.

2. *Boiling Test.* Make the ordinary coagulation test according to the directions given under "Albumin," p. 831. If no coagulable protein is found, allow the boiled urine to stand and note the gradual appearance, in the cooled fluid, of a flaky precipitate of proteose.

3. *Detection of Bence-Jones Protein.* Heat the suspected urine very gently, carefully noting the temperature. At as low a temperature as 40° C. a turbidity may be observed, and as the temperature is raised to about 60° C. a flocculent precipitate forms and clings to the sides of the test tube. If the urine is now acidified *very slightly* with acetic acid and the temperature further raised to 100° C., the precipitate at least partly disappears. Filter while boiling hot. The precipitate returns on cooling the tube. The addition of a few drops of dilute (1 per cent) calcium chloride solution to the urine before testing frequently improves the response to this test.

This property of precipitating at so low a temperature and of dissolving at a higher temperature is typical of Bence-Jones protein and may be used to differentiate it from all other forms of protein material occurring in the urine.

4. *Osgood-Haskins Test for Bence-Jones Protein.* See Exp. 5, p. 831.

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[11] If it is considered desirable to test for peptone the proteose may be removed by saturation with $(NH_4)_2SO_4$ according to the directions given on p. 197 and the filtrate tested for peptone by the biuret test.

NUCLEOPROTEIN

There has been considerable controversy as to the proper classification for the protein material which forms the nubecula of normal urine. By different investigators it has been called mucin, mucoid, phosphoprotein, nucleoalbumin, and nucleoprotein. Of course, these terms are not synonymous. Mucin and mucoid are glycoproteins and hence contain no phosphorus (see p. 185), whereas phosphoproteins and nucleoproteins are phosphorized compounds. It may possibly be that both these forms of protein, i.e., the glycoprotein and the phosphorized type, occur in the urine under certain conditions (see p. 810). In this connection we will use the term *nucleoprotein*. The pathological conditions under which the content of nucleoprotein is increased include all affections of the urinary passages and in particular pyelitis, nephritis, and inflammation of the urinary bladder.

TESTS FOR NUCLEOPROTEIN

1. *Detection of Nucleoprotein.* Place 10 ml. of urine in a small beaker, dilute it with three volumes of water to prevent precipitation of urates, and make the reaction very strongly acid with acetic acid. If the urine becomes turbid it is an indication that nucleoprotein is present.

If the urine under examination contains albumin the greater portion of this substance should be removed by boiling the urine before testing it for the presence of nucleoprotein.

2. *Tannic Acid Precipitation Test (Ott).* Mix 25 ml. of the urine with an equal volume of a saturated solution of sodium chloride and slowly add Almén's reagent.[12] In the presence of nucleoprotein a voluminous precipitate forms.

BLOOD

The pathological conditions in which blood occurs in the urine may be classified under the two divisions *hematuria* and *hemoglobinuria*. In hematuria we are able to detect not only the hemoglobin but the unruptured corpuscles as well, whereas in hemoglobinuria the pigment alone is present. Hematuria is brought about through blood passing into the urine because of some lesion of the kidney or of the urinary tract below the kidney. Hemoglobinuria is brought about through hemolysis, i.e., the rupturing of the stroma of the erythrocyte and the liberation of the hemoglobin. This may occur in malaria, typhoid, yellow fever, hemolytic jaundice, and other diseases. It may also occur as the result of a burn covering a considerable area of the body, through the action of certain hemolytic poisons, or as a result of transfusion with incompatible blood.

DETECTION OF BLOOD

1. Benzidine Reaction. This is one of the most delicate of the reactions for the detection of blood. Different benzidine preparations vary greatly in their sensitiveness, however. Inasmuch as benzidine solutions change readily upon contact with light, it is essential that they be kept in a dark place.

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[12] See Appendix.

The test is performed as follows: To 3 ml. of a saturated solution of benzidine in glacial acetic acid[13] add 2 ml. of urine and 1 ml. of 3 per cent hydrogen peroxide. A blue or green color indicates a positive test. The following test is more delicate and specific.

Confirmatory Test. **To 10 ml. of urine add 1 to 2 drops of glacial acetic acid and extract by shaking with 5 ml. of ether. Pour the ether extract into a small evaporating dish. Put on a hot water bath (with flame turned out) and evaporate to dryness. To the residue add a few drops of water, a drop of benzidine solution, and a drop of hydrogen peroxide. A blue or green color indicates blood.**

Often when urines containing a small amount of blood are tested by the direct procedure, the mixture is rendered so turbid as to make it difficult to decide as to the presence of a faint green color. The sensitiveness of the benzidine reaction is greater when applied to aqueous solutions than when applied to the urine.

For modifications of this test and further discussion see page 484.

2. *Guaiac Test.* Place 5 ml. of urine[14] in a test tube and by means of a pipet introduce a freshly prepared alcoholic solution of guaiac (strength about 1:60) into the fluid until a turbidity results, then add old turpentine or hydrogen peroxide, drop by drop, until a blue color is obtained.

This test is also much more delicate when applied to the acid-ether extract. The test is positive both before and after boiling the urine for 15 to 20 seconds. Pus does not respond after boiling. Old, partly putrefied pus gives the test even without the addition of hydrogen peroxide or old turpentine, whereas fresh pus responds upon the addition of hydrogen peroxide. See the discussion on p. 480 and the test on p. 484.

3. *Spectroscopic Examination.* Submit the urine to a spectroscopic examination according to the directions given on p. 494, looking especially for the absorption bands of oxyhemoglobin and methemoglobin (see Fig. 110, p. 473).

MYOGLOBIN

This heme pigment, derived from muscular tissue, is found in the urine after extensive destruction of muscular tissue, as from crushing injuries. Urine containing myoglobin resembles that containing blood; it may be smoky dark brown or red, and will give a positive benzidine test. Red cells, however, are notably absent, and the sediment may contain brown pigmented casts. Myoglobin has a molecular weight only about one-fourth that of hemoglobin (see Chapter 22 for further discussion) which probably explains its ready diffusibility through the kidney membranes. The appearance of myoglobin in the urine is usually associated with concomitant kidney damage, both clinically and experimentally, but whether this has any direct connection with the presence of myoglobin is not known.

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[13] Glacial acetic acid is preferable, but, if it is not available, alcohol acidified with acetic acid may be used.

[14] Alkaline urine should be made slightly acid with acetic acid as the blue end-reaction is very sensitive to alkali.

PUS

Pus may be present in the urine in inflammatory affections of various types. Such a condition is termed *pyuria*. Albumin always accompanies the pus. In catarrh of the urinary bladder and in inflammation of the urethra or of the pelvis of the kidney, pus is particularly apt to be present in the urine. If a urine of high pus concentration is voided it may indicate the rupturing of an abscess in some part of the genitourinary tract. Pus may be detected by one of the procedures given below.

TESTS FOR PUS

1. ***Microscopical Detection of Pus.*** The characteristic form elements of pus are leukocytes. They may occur in very small number in normal urine. Examine the urine (centrifuged if necessary) under the microscope. Any considerable number of pus corpuscles indicates a pathological urine. In *acid urine* the pus corpuscles appear as round, colorless cells, composed of refractive, granular protoplasm. Sometimes they may exhibit ameboid movements, particularly if the slide containing them be warmed slightly. They are nucleated (one or more nuclei), the nuclei being clearly visible only upon treating the cells with water, acetic acid, or some other suitable reagent. In *alkaline urine* the pus corpuscles are often degenerated. They may occur as swollen, transparent cells, which exhibit no granular structure. If the degeneration has proceeded far enough the nuclei fade and the cell disintegrates and only debris remains.

Sometimes it is almost impossible to differentiate between pus corpuscles and certain types of epithelial cells. In such a case apply the following chemical test.

2. ***Guaiac Test.*** **This test is not specific for pus, but is given by certain other substances and particularly by blood (see p. 835). Perform the test as follows: Acidify the urine (if alkaline) with acetic acid, filter and add tincture of guaiac to the sediment on the paper. If the pus is old and partly putrefied, it will give a blue color. If no blue color is secured, add old turpentine, or hydrogen peroxide, drop by drop. A blue color formed only under these conditions indicates fresh pus.**

As a control test boil some of the urine (or sediment) for 15 to 20 seconds and repeat the test. Pus does not respond after boiling. In the case of blood the test is positive both before and after boiling.

BILE

Both the pigments and the acids of the bile may be detected in the urine under certain pathological conditions. A urine containing bile may be yellowish green to brown in color and when shaken foams readily. The staining of the various tissues of the body through the absorption of bile due to occlusion of the bile duct is a prominent symptom of the condition known as icterus or jaundice. Bile is always present in the urine under such conditions unless the amount of bile reaching the tissues is extremely small.

TESTS FOR BILE PIGMENTS

Practically all of the tests for bile pigments are based on the oxidation of the pigment by a variety of reagents with the formation of a series of colored derivatives. For detailed chemistry of these tests see the chapter on bile. A simple though satisfactory test for bile consists in shaking the urine in a test tube and observing the yellow foam.

1. *Gmelin's Test.* To about 5 ml. of concentrated nitric acid in a test tube add an equal volume of urine carefully so that the two fluids do not mix. At the point of contact note the various colored rings; green, blue, violet, red, and reddish yellow. (Fuming yellow nitric acid gives the best results.)

2. *Rosenbach's Modification of Gmelin's Test.* Filter 5 ml. of urine through a small filter paper. Introduce a drop of concentrated nitric acid into the cone of the paper and observe the succession of colors as given in Gmelin's test.

3. *Harrison Spot Test.*[15] S & S filter paper No. 470 is impregnated with a 10 per cent barium chloride solution and dried. Dip the dry paper into the urine for 10 seconds and add a drop of Fouchet's reagent[16] at the surface line. A *green* color indicates bilirubin. This test has been modified by Hawkinson, Watson and Turner.[17]

TESTS FOR BILE ACIDS

1. *Furfural-H_2SO_4 Test* (*Mylius*). To 5 ml. of urine in a test tube add 3 drops of a very dilute (1:1000) aqueous solution of furfural. Now incline the tube, run about 2 to 3 ml. of concentrated sulfuric acid carefully down the side, and note the red ring at the point of contact. Upon slightly agitating the contents of the tube the whole solution gradually assumes a reddish color. As the tube becomes warm, it should be cooled in running water in order that the temperature may not rise above 70° C.

It is claimed that this test is not satisfactory in the presence of protein and chromogenic substances which yield interfering colors with sulfuric acid.

2. *Surface Tension Test* (*Hay*). This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. The test is performed as follows: Cool about 10 ml. of fresh urine in a test tube to 17° C. or lower, and sprinkle a little finely pulverized sulfur upon the surface of the fluid. The presence of bile acids is indicated if the sulfur sinks to the bottom of the liquid, through the surface film. Compare with a control tube of normal urine known to be free from bile. Urines preserved with thymol may respond positively to this test.

THE ACETONE BODIES

The *acetone* (or *ketone*) *bodies* include the compounds acetoacetic (or diacetic) acid, β-hydroxybutyric acid, and acetone. The chemical relationship between these various acetone bodies is as indicated below; acetoacetic acid and β-hydroxybutyric acid are primary products, the latter probably being formed by reduction of the former. Acetone, however, is a decomposition product of acetoacetic acid and is probably not produced as such within the body, although it is invariably found there when the other two acetone bodies are present.

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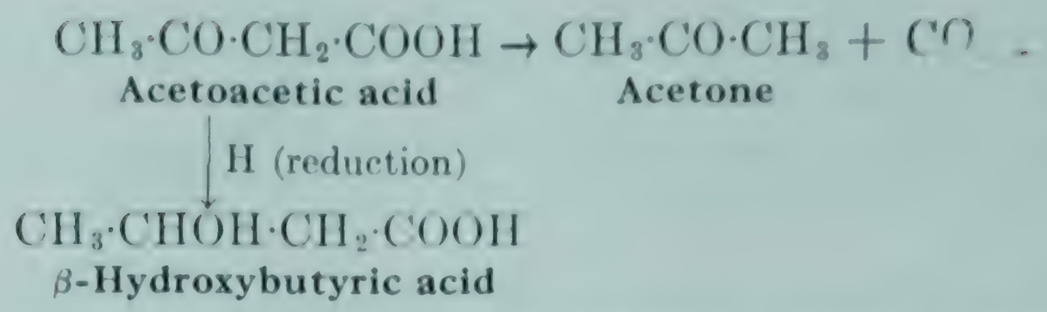
[15] Harrison: *Chemical Methods in Clinical Medicine*, London, J. & A. Churchill, Ltd., 1937.

[16] Fouchet's reagent is 25 per cent trichloroacetic acid containing 0.9 per cent ferric chloride.

[17] *J. Am. Med. Assoc.*, **129**, 514 (1945).



$$\underset{\textbf{Acetoacetic acid}}{CH_3\cdot CO\cdot CH_2\cdot COOH} \rightarrow \underset{\textbf{Acetone}}{CH_3\cdot CO\cdot CH_3} + CO$$

$$\Big\downarrow \text{H (reduction)}$$

$$\underset{\boldsymbol{\beta}\textbf{-Hydroxybutyric acid}}{CH_3\cdot CHOH\cdot CH_2\cdot COOH}$$

Acetoacetic acid and β-hydroxybutyric acid appear to be either intermediate products in the breakdown of fatty acid chains or secondary combinations of 2-carbon fragments formed in this breakdown or closely related to it. Under normal conditions the fatty acids in the animal body are oxidized completely to carbon dioxide and water and intermediate products do not appear to any great extent in the blood or urine. In certain abnormal conditions, however, the ketone bodies accumulate in the blood (*ketonemia*) and are excreted in the urine (*ketonuria*); this general condition is known as a *ketosis*. Ketosis is apparently always associated with some abnormality of carbohydrate metabolism. It is still a disputed question whether ketosis results from the failure on the part of the animal body to oxidize completely the ketone bodies produced normally, or from an overproduction of ketone bodies by the organism in an attempt to meet fuel requirements not supplied by carbohydrates. Ketosis occurs in fasting or during carbohydrate deprivation and in such conditions disappears when carbohydrate is fed. Pathologically it is most severe in diabetes mellitus, in which disease the accumulation of the ketone body acids is largely responsible for the development of diabetic acidosis. Diabetic urine during ketonuria is often high in ammonia which is formed by the body to combat acidosis. Diabetic ketosis is alleviated by insulin treatment. Ketosis also occurs in the acetonemic vomiting of childhood, and frequently in pregnancy, fevers, ether and chloroform anesthesia, malnutrition, prolonged feeding of a carbohydrate-poor diet high in meat and fat, and many other conditions. (See also Chapters 31 and 33.)

Acetone, $CH_3\cdot CO\cdot CH_3$. Acetone when pure is a liquid which possesses a characteristic aromatic fruitlike odor, boils at 56° to 57° C., and is miscible with water, alcohol, or ether in all proportions. It reacts with alkali and iodine to yield iodoform. With sodium nitroprusside in alkaline solution it gives a red compound. With salicylic aldehyde, in strongly alkaline solution, it forms a red to orange condensation compound. It forms a nonvolatile compound with bisulfites.

Acetoacetic Acid, $CH_3\cdot CO\cdot CH_2\cdot COOH$. In the pure state acetoacetic acid is a colorless liquid, miscible with water, alcohol, and ether, in all proportions. Its solution gives a Bordeaux-red color with ferric chloride (not given by acetone). With nitroprusside in alkaline solution it gives a permanganate color, similar to that given by acetone but many times more intense on an equivalent basis. In aqueous solution acetoacetic acid decomposes (most readily on warming in acid solution) to yield acetone. Hence after heating or long standing it gives the tests for acetone, and is probably never found in urine without the concomitant presence of acetone. It is always determined quantitatively as acetone. Acetone and acetoacetic acid may be separated by blowing off the acetone with a current of air under the proper conditions.

β-Hydroxybutyric Acid, $CH_3 \cdot CHOH \cdot CH_2 \cdot COOH$. Ordinary β-hydroxybutyric acid is an odorless, transparent, levorotatory syrup easily soluble in water, alcohol, and ether; it may be obtained in crystalline form. It does not respond to the tests for acetone or acetoacetic acid unless it is first oxidized, when it yields acetoacetic acid and, on decomposition, acetone. β-Hydroxybutyric acid in acid urines acts as a urinary antiseptic. Hence ketogenic diets may be useful in cases of infection of the urinary tract.

Occurrence of Acetone Bodies in Urine. Total ketone bodies are found in normal urine to the extent of about 20 mg. (expressed as acetone) in 24 hours, but this amount is variable. Of the total, one-half or more is usually in the form of β-hydroxybutyric acid, but this ratio is also variable. Pathologically, values of from 0.02 to 6 g. or more per day of combined acetone and acetoacetic acid have been observed. β-Hydroxybutyric acid has been found in the urine in severe diabetes in amounts of 50 to 100 g. or over. In such conditions the β-hydroxybutyric acid may constitute 60 to 80 per cent of the total acetone bodies. In rare cases excretion of large amounts of β-hydroxybutyric acid may occur with low acetone output.

TESTS FOR ACETONE BODIES

The three acetone bodies practically always occur together in the urine and have essentially the same significance. The usual tests for ketonuria are tests for acetone or acetoacetic acid or both, since a distillate obtained by heating urine will contain acetone derived by decomposition of acetoacetic acid as well as preformed acetone, and the nitroprusside reaction applied to urine will detect either compound, although with quite different relative sensitivity. There is no satisfactory simple direct test for β-hydroxybutyric acid in urine. The ferric chloride test for acetoacetic acid is not given by acetone, but the test is neither sensitive nor specific and cannot be recommended for clinical work, except perhaps as a confirmatory test. It must be made on the urine directly, and not on a distillate.

The nitroprusside test (particularly the Rothera version) is much more sensitive (at least five or more times) to acetoacetic acid than to acetone; hence when applied to urine directly the result will depend largely on the relative amounts of these two substances present. Fresh urine may give a positive test; the same urine on standing and after decomposition of the acetoacetic acid to acetone may give a negative test. In a distillate, where both compounds are present as acetone, the reaction is sometimes fainter than in the original urine. The test is not entirely specific for the acetone bodies, but when positive it is strongly indicative of their presence. It is probably the most widely used test for ketonuria in clinical work, and many variations in technique have been proposed; nevertheless it cannot be regarded as entirely satisfactory.

The iodoform and salicylic aldehyde tests react with acetone but not directly with acetoacetic acid, and because of interfering substances in urine must be carried out on acetone separated from the urine by distillation or otherwise. The iodoform test on a distillate is quite sensitive and may be of value in suggesting the presence of very small amounts of acetone bodies, but it is not entirely specific for acetone (alcohol for example also gives a positive test) and it is less satisfactory for clinical work than the other tests described here. The salicylic aldehyde test, carried out as described here on acetone separated from the urine, is probably the most generally satisfactory test for ketonuria.

TESTS FOR ACETONE AND ACETOACETIC ACID

1. *Isolation from the Urine*. The tests for acetone are more satisfactory if the acetone is separated from the urine by distillation and the tests applied to the distillate. Introduce into a small distilling bulb 10 ml. (or more) of the urine to be tested, and acidify. Distil off about 2 ml. of liquid (containing most of the acetone) into a test tube. A condenser is not necessary but the tube should be kept cool. In the distillation any acetoacetic acid present is decomposed and the acetone from this source also passes into the distillate. Try either of the two following tests on the distillate.

2. *Iodoform Test* (*Lieben*). To 2 ml. of distillate add 3 to 5 drops of 10 per cent NaOH and then Lugol's iodine solution drop by drop to a faint yellow. Let stand at room temperature if necessary. A definite turbidity changing to a yellow precipitate of iodoform should be noted. The odor is characteristic as is also the crystalline form viewed under the microscope (see p. 68).

This test is given by alcohol which may be formed by fermentation in diabetic urines. Alcohol, however, reacts much more slowly. If ammonia is used, usually 5 to 10 drops, instead of NaOH (Gunning's test) the reaction is more specific for acetone but is less delicate.

3. *Nitroprusside Test* (*Legal*). Try this test on both the original urine and the distillate. To 2 ml. of liquid add a few drops of a freshly prepared 5 per cent aqueous solution of sodium nitroprusside. Make alkaline with NaOH. A red color indicates acetone. If the test is made directly on urine a red color is given by creatinine which, however, disappears on the addition of acetic acid. A modification of this test in quite general use is as follows: to a few ml. of urine add a few drops of the nitroprusside solution and mix. Add concentrated ammonium hydroxide carefully down the side of the tube so as to form a layer over the sample. A purple (not brown) ring at the zone of contact indicates the presence of acetone bodies.

4. *Nitroprusside Test* (*Rothera*).[18] Saturate 20 ml. of urine with ammonium sulfate by shaking with the crystals in a test tube. Add 2 to 3 drops of concentrated NH_4OH and a few drops of a freshly prepared 5 per cent solution of sodium nitroprusside and shake. A positive test is indicated by the development of a permanganate tinge which gradually deepens. A brown color is not a positive test. A quick strong reaction indicates about 0.25 per cent acetoacetic acid while a slow weak reaction is given by 0.0005 per cent acetoacetic acid. A faint test has less significance than the ferric chloride test[19] because of the delicacy of the reaction. The test is given by acetone also but is much less delicate for this substance.

5. *Salicylic Aldehyde Test* (*Behre*).[20] In this test a previous distillation is unnecessary since the acetone, preformed and from acetoacetic acid, is dis-

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[18] The dry ingredients of the Rothera test for acetone are available commercially as a powder ("Acetone Test," obtainable from the Denver Chemical Mfg. Co., Inc., New York City). In use, a few drops of urine are placed on a small amount of the powder. A purple color indicates acetone.

[19] See Exp. 6.

[20] Behre: *J. Lab. Clin. Med.*, 13, 770 (1928). Also personal communication.

tilled in the test tube onto the reagents. Place 3 ml. of urine in a clean test tube and 3 ml. of distilled water as a control in another tube, and add 1 drop of 1:1 sulfuric acid to each. Prepare two small thin squares of cotton and in the center of each place a drop of salicylic aldehyde (or of an alcoholic solution of salicylic aldehyde[21]) and two drops of a saturated solution of potassium hydroxide. These reagents solidify to form a yellow disk. When they have solidified, invert a cotton square over each test tube and push in slightly so that the spot of reagents faces down toward the fluid in the tube but does not touch the sides of the tube. Place both tubes upright in boiling water for eight minutes. Remove the cotton and examine the spots. The presence of acetone or acetoacetic acid in the urine is indicated by a pink to deep rose coloration of the spot, as compared with the yellow color of the blank test. The color from acetone bodies deepens on standing and in doubtful cases should be examined after a few minutes. Familiarity with the test makes it possible to estimate roughly the amount of total acetone present.

6. *Ferric Chloride Test for Acetoacetic Acid*[22] (*Gerhardt*). To 5 ml. of urine in a test tube add ferric chloride solution, drop by drop, until no more precipitate forms. In the presence of acetoacetic acid, a Bordeaux-red color is produced; this color may be somewhat masked by the precipitate of ferric phosphate, in which case the fluid should be filtered.

A positive result from the above manipulation simply indicates the possible presence of acetoacetic acid. Before making a final decision regarding the presence of this substance make the two following control experiments:

***a.* Place 5 ml. of urine in a test tube, small beaker, or Erlenmeyer flask and boil it vigorously for three to five minutes. Cool the vessel and, with the boiled urine, make the test as given above. Compare with the test on the unboiled sample. As has been already stated, acetoacetic acid yields acetone upon decomposition and acetone does not give a Bordeaux-red color with ferric chloride. By boiling as indicated above, therefore, any acetoacetic acid present would be decomposed into acetone and carbon dioxide and the test upon the resulting fluid would be negative. If positive, the color is due to the presence of substances other than acetoacetic acid.**

***b.* Place 5 ml. of urine in a test tube, acidify with H_2SO_4, to free acetoacetic acid from its salts, and carefully extract the mixture with ether by shaking. If acetoacetic acid is present, it will be extracted by the ether. Now remove the ethereal solution, evaporate it to dryness, dissolve the residue in 1 to 2 ml. of water, and add 3 to 5 drops of 3 per cent ferric chloride. Acetoacetic acid is indicated by the production of the characteristic Bordeaux-red color.**

This color disappears spontaneously in 24 to 48 hours. Such substances as antipyrine, acetophenetidine, salicylic acid, salicylates, sodium acetate, thiocyanates, and thallin yield a similar red color under these conditions; when due to the presence of any of these substances, however, the color does not disappear spontaneously but may remain for days. Many of these disturbing substances are soluble in benzene or chloroform and may be removed from the urine by this means before extracting with ether as above. Acetoacetic acid is insoluble in benzene or chloroform.

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[21] Eastman's technical grade or Eimer and Amend's Acid Salicylous, Synthetic, are usually satisfactory for use without dilution. If blackening appears on the cotton during the heating a solution of 1 part salicylic aldehyde in 1 part methyl or ethyl alcohol should be used.

[22] To prepare a solution which may be added to urine, if urines containing this acid are not available for student work, proceed as follows: Treat 13 g. of ethyl acetoacetate with 500 ml. of 0.2 N sodium hydroxide. Allow to stand for 48 hours to hydrolyze the ester. In preparing urine for tests add 1 part of this solution to 10 parts of urine.

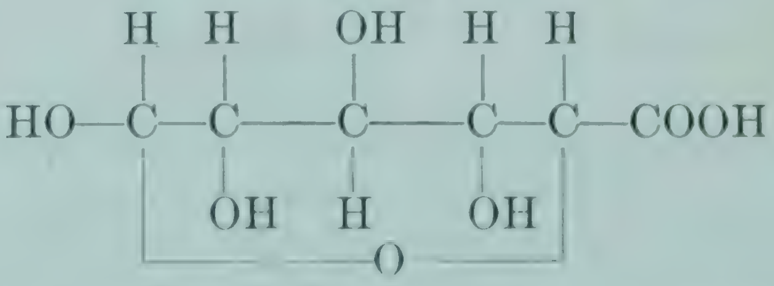
TESTS FOR β-HYDROXYBUTYRIC ACID

1. Black's Reaction. **Inasmuch as the urinary pigments as well as any contained sugar or acetoacetic acid will interfere with the delicacy of this test when applied to the urine directly, the following preliminary procedure is necessary: Concentrate 10 ml. of the urine under examination to one-third or one-fourth of its original volume in an evaporating dish at a gentle heat. Acidify the residue with a few drops of concentrated hydrochloric acid, add sufficient plaster of Paris to make a thick paste, and allow the mixture to stand until it begins to set. It should now be stirred and broken up in the dish by means of a stirring rod with a blunt end. Extract the porous meal thus produced twice with ether by stirring and decantation. Any β-hydroxybutyric acid present will be extracted by the ether. Evaporate the ether extract spontaneously or on a water bath, dissolve the residue in water, and neutralize it with barium carbonate. To 5 to 10 ml. of this neutral fluid in a test tube add 2 to 3 drops of ordinary commercial acid hydrogen peroxide. Mix by shaking and add a few drops of Black's reagent.[23] Permit the tube to stand and note the gradual development of a rose color which increases to its maximum intensity and then gradually fades.[24]**

In carrying out the test care should be taken to see that the solution is cold and approximately neutral and that a large excess of hydrogen peroxide and Black's reagent are not added. In case but little β-hydroxybutyric acid is present the color will fail to appear or will be but transitory if the oxidizing agents are added in too great excess. It is preferable to add a few drops of the reagent and at intervals of a few minutes repeat the process until the color undergoes no further increase in intensity. One part of β-hydroxybutyric acid in 10,000 parts of the solution may be detected by this test.

2. Polariscopic Examination. **Subject some of the urine (free from protein) to the ordinary fermentation test (see p. 827). This will remove glucose and fructose, which would interfere with the polariscopic test. Now examine the fermented fluid in the polariscope and if it is levorotatory the presence of β-hydroxybutyric acid is indicated. This test is not absolutely reliable, however, since conjugate glucuronates are also levorotatory and withstand fermentation.**

GLUCURONIC ACID



Glucuronic acid does not occur free in urine, but is found in combinations known as *conjugate glucuronates* or *glucuronides* with a wide variety of compounds under both normal and pathological conditions or after administration of such compounds for medicinal or experimental purposes. There are two general types of conjugate glucuronates, the *glycoside* type and the *ester* type, both of which involve linkage with glucuronic acid through the OH group on carbon atom number 1 of the cyclic structure. The glycoside type involves the OH group of aliphatic or aromatic alcohols, e.g., phenol, naphthol, borneol, etc., while the ester type involves reaction with the COOH group of such compounds as benzoic acid, phenylacetic acid, etc. Free glucuronic acid is as powerful a reducing substance as glucose, and its quantitative determination may be based upon this fact; ester-type glucuronides likewise show direct reducing properties, probably due to concomitant hydrolysis, while the glycoside type is nonreducing until hydrolyzed. Conjugate glucuronates may therefore interfere with the reducing tests for sugar in urine under certain conditions, but they may be readily distinguished from glucose because of their nonfermentability with yeast. While glucuronic acid is dextrorotatory, the glucuronides as a class are levorotatory; this also serves for distinction from glucose in urine.

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[23] Made by dissolving 5 g. of ferric chloride and 0.4 g. of ferrous chloride in 100 ml. of water.

[24] This disappearance of color is due to the further oxidation of the acetoacetic acid.

The total glucuronic acid content of normal urine appears to approximate 0.5 to 1.0 g. per day; earlier estimates were considerably lower than this, probably because the methods were based on the mistaken assumption that all conjugate glucuronates are soluble in ether. The glucuronides of normal urine include combinations with phenol, indoxyl, skatoxyl, and the estrogenic hormones. Urine glucuronide content may be greatly increased by the administration of a variety of compounds, such as antipyrine, acetylsalicylic acid (aspirin), borneol, camphor, chloral hydrate, menthol, morphine phenolphthalein, turpentine, and practically all of the sulfonamides (except possibly sulfanilamide). Experimentally, certain carcinogenic substances or derivatives are ultimately excreted in part as glucuronides. Glucuronic acid formation and conjugation appear to take place in the liver. The origin of glucuronic acid is obscure, although it is known to be an end product of the polysaccharide-splitting action of the enzyme hyaluronidase.

TESTS FOR GLUCURONATES

1. *Naphthoresorcinol Reaction* (*Tollens*). Introduce 5 ml. of urine in a test tube and add 0.5 to 1 ml. of a 1 per cent solution of naphthoresorcinol in 95 per cent alcohol, and 5 ml. of concentrated hydrochloric acid. Raise the temperature gradually to the boiling point and boil for one minute, shaking the tube continuously. Stand the tube aside four minutes, then cool under the tap. Extract with an equal volume of ether (preferably peroxide-free). Glucuronates are indicated by the ether extract assuming a violet-red color. The spectroscope shows this extract to possess an absorption band in the green to yellow region of the spectrum. The peak absorption is at approximately 570 mμ.

2. *Preparation of Glucuronic Acid* (*Method of Quick*).[25] Give 5 g. of pulverized borneol daily to each of several dogs. Collect the urine, acidify with acetic acid, and add lead acetate. Most of the coloring matter is carried down. Filter, heat the filtrate to boiling, and add an excess of zinc acetate. Filter off the precipitate and wash with hot water until no more coloring matter is extracted. This is practically pure zinc borneol glucuronic acid. About 1 g. is obtained for each g. of borneol given.

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[25] Quick: *J. Biol. Chem.*, 74, 331 (1927).

Dissolve the finely powdered zinc salt in hot 3.5 N sulfuric acid, using about 140 ml. for each 100 g. of the salt. When completely dissolved, cool rapidly and put in an icebox for several hours. Filter off the crystals of borneol glucuronic acid, wash with a little cold water, and dry in the air.

Dissolve 100 g. of borneol glucuronic acid in 1,500 ml. of 0.2 N sulfuric acid and boil for three hours beneath a reflux condenser. Filter and treat the hot filtrate with sufficient barium hydroxide to precipitate the last traces of sulfuric acid. Allow the mixture to settle. Siphon off the supernatant fluid, and complete the separation by centrifuging. Concentrate the solution under diminished pressure to a syrupy consistency, and let it stand to crystallize. Filter off the crystals and wash them with a small amount of alcohol to remove the pigment. This is a mixture of glucuronic acid and its lactone. Treat 4 g. of the product with 200 ml. of 95 per cent alcohol and set aside for 12 hours. Repeat twice, using 100-ml. portions of alcohol. The residue should be glucuronic acid of 99 per cent purity. To obtain the pure lactone, dissolve some of the mixture of acid and lactone in hot glacial acetic acid, allow the solution to cool, and recrystallize from hot water.

Glucuronic acid is a syrupy liquid, readily soluble in water and slightly soluble in alcohol. When the aqueous solution is boiled, evaporated, or even allowed to stand at the ordinary temperature, the acid loses the elements of water and yields the anhydride or lactone. It is a strong organic acid ($K > 1 \times 10^{-3}$).

Glucuronic anhydride, $C_6H_8O_6$, forms monoclinic tables or needles, having a sweet taste, and m.p. 160° when heat is gradually applied, or at 170° to 180° when heated rapidly. The anhydride is insoluble in alcohol, but dissolves readily in water to form a dextrorotatory solution, $[\alpha]_D = +19.25°$. The solution prevents the precipitation of cupric ion by alkalies, and powerfully reduces Fehling's solution, the copper-reducing power being 98.8, compared with glucose as 100.

Glucuronic acid itself is dextrorotatory ($[\alpha]_D = +36°$), but many of its compounds are levorotatory. It shows mutarotation with an initial value of $[\alpha]_D^{20} = +16°$. It reduces Fehling's solution on heating, and precipitates the metals from hot alkaline solutions of silver, mercury, and bismuth.

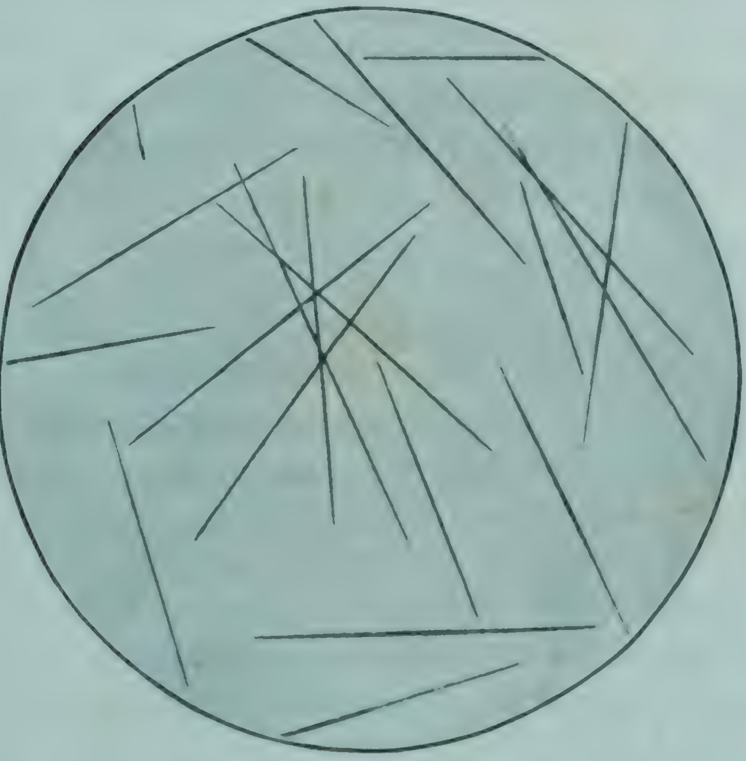

Fig. 228. Pentosazone Crystals.

Isolated and purified in the senior author's laboratory by Dr. B. L. Fleming. For color of crystals see Plate II, opposite p. 63.

PENTOSES

There are two distinct types of pentosuria, viz., (1) alimentary pentosuria, resulting from the ingestion of large quantities of pentose-rich fruits such as prunes, cherries, grapes, or plums, and fruit juices, in which condition the pentoses appear only temporarily in the urine; and (2) the chronic form of pentosuria, in which the output of pentoses bears no relation whatever to the quantity and nature of the pentose content of the food eaten. Pentosuria is apparently a harmless abnormality; it is definitely known that pentosuria bears no relation to diabetes mellitus and there is no generally accepted theory to account for the occurrence of the chronic form of pentosuria. The pentose detected most frequently in the urine in chronic pentosuria is L-xyloketose. L-Arabinose has been found in cases of alimentary pentosuria. For pentosazone crystals, see Fig. 228.

TESTS FOR PENTOSE

1. *Benzidine Reaction* (*Tauber*).[26] To 0.1 ml. of the urine in a test tube, add 0.5 ml. of a 4 per cent solution of benzidine in glacial acetic acid. Heat to vigorous boiling, cool under the cold-water tap, and add 1 ml. of water. The presence of pentose is indicated by the immediate appearance of a pink to red color. If pentoses are absent, the mixture has a yellowish-brown color.

This test is highly specific for pentoses in the free form but is not given by gum arabic, nucleic acids, or riboflavin in which the pentose is combined. Normal and abnormal constituents of urine do not interfere. Too large amounts of protein may be removed from pathological urine by mixing with an equal volume of 10 per cent trichoroacetic acid solution, warming to 95°, and filtering. The test is applied to the filtrate. This test is said to detect the presence of aldopentoses but not ketopentoses. The 4 per cent benzidine solution is stable for four days.

2. *Orcinol-Hydrochloric Acid Reaction* (*Bial*). To 5 ml. of Bial's reagent[27] in a test tube, add 2 to 3 ml. of urine and heat the mixture gently until the first bubbles rise to the surface.[28] Immediately or upon cooling the solution becomes green and a flocculent precipitate of the same color may form.

This test is believed to be more accurate than the original orcinol test. It is claimed that urines containing menthol, kreosotal, etc., respond to the old orcinol reaction, but not to Bial's. If so desired the osazone of the pentose (see Fig. 228) may be formed, then distilled with hydrochloric acid and the distillate tested by Bial's test (Jolles).

3. *Phloroglucinol-Hydrochloric Acid Reaction* (*Tollens*). To equal volumes of urine and hydrochloric acid (sp. gr. 1.09) add a little phloroglucinol and heat the mixture on a boiling water bath. Pentose, galactose, or glucuronic acid will be indicated by the appearance of a red color. To differentiate between these compounds examine by the spectroscope and look for the absorption band in the yellow region of the spectrum given by pentoses and glucuronic acid, and then differentiate between the two latter compounds by the melting points of their osazones.

4. *White-Green Reaction*.[29] To the urine in a test tube add two drops of acetic acid and boil. Add Norit, shake two minutes and filter. To 2 ml. of filtrate add 2 ml. of acetic acid and 5 drops of redistilled aniline. Heat to boiling, allow to stand 2 minutes then cool and extract with 2 ml. of chloro-

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[26] Tauber: *Proc. Soc. Exptl. Biol. Med.*, **37**, 600 (1937).

[27] See Appendix.

[28] The test may also be performed by adding the urine to the *hot* reagent. No further heating should be necessary if pentose is present.

[29] White and Green: *Trans. Roy. Soc. Section V, Biological Sciences, Third Series*, **26**, 145 (1932).

form. In the presence of pentose the chloroform extract becomes bright red. Glucose and galactose give a green color, fructose a pale yellow, and glucuronate a very pale yellow.

LACTOSE

Lactose is rarely found in the urine except when excreted by women during pregnancy, during the nursing period, or soon after weaning, i.e., when the mammary glands are actively functioning. Lactosuria has no pathological significance, representing as it does simply the excretion of a sugar which has found its way into the blood stream by some means and which is not utilizable as such by the organism.[30] It is nevertheless important to be able to identify the condition of lactosuria and to distinguish it from glucosuria, otherwise serious misinterpretation of laboratory data may result. Lactose in urine is readily distinguished from glucose by its lack of fermentability with ordinary yeast; qualitative or better quantitative determination of reducing power before and after exhaustive treatment with yeast is of value in this connection, particularly for the detection of a concomitant glucosuria. The characterization of lactose as its typical osazone is not ordinarily attended with much success in urine; better results may be obtained by adsorption of the lactose on charcoal prior to testing, as described below. The mucic acid test, which is specific for lactose and galactose, may be used to identify lactose in urine, but interpretation of results may be obscured by the presence of other insoluble crystalline substances.

TESTS FOR LACTOSE

1. *Fermentation Test*. Ferment the urine as in Exp. 3(a) or 3(b), p. 827. If nonfermentable reducing sugar is found it is very probably lactose, especially if the patient be pregnant or lactating. In rare cases it may be pentose. Lactose may be distinguished from pentose by proper application of Tauber's test (p. 845), the mucic acid test, or the osazone test.

2. *Osazone Test* (*According to Cole*). Shake 25 ml. of urine with 1 g. of Merck's medicinal charcoal, boil a few seconds, cool thoroughly, and shake at intervals for 10 minutes. Filter through a small paper or use a filter pump. Let drain completely. Transfer the charcoal to a dish containing 10 ml. of water and 1 ml. of glacial acetic acid. Boil for about 10 seconds and filter hot into a tube containing as much phenylhydrazine hydrochloride as will lie on a quarter and twice as much sodium acetate. Mix and heat on a boiling water bath for 45 minutes. Remove and let stand for at least an hour. Look for "hedgehog crystals" of lactosazone. Glucose, if present in significant amount, will be adsorbed by the charcoal to some extent and come through along with the lactose to give glucosazone crystals. The value of the charcoal separation is chiefly to increase the concentration of lactose relative to glucose and to facilitate the obtaining of characteristic lactosazone crystals.

3. *Mucic Acid Test*. Transfer 50 ml. of urine to a 150-ml. beaker and add 12 ml. of concentrated HNO_3. Heat on a boiling water bath until the volume is reduced to about 10 ml. Cool. Add 10 ml. of water and let stand overnight. A fine white precipitate of mucic acid will form if lactose or galactose is

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[30] If any lactose is excreted into the intestinal tract and there hydrolyzed into glucose and galactose, these products are capable of absorption and utilization by the body.

present. Examine the crystals under the microscope (see Fig. 19). Other reducing sugars do not give this test. Lactose and galactose may be differentiated by Tollens' test (see tests for pentose).

GALACTOSE

Galactose has occasionally been detected in the urine, and in particular in that of nursing infants afflicted with a deranged digestive function. It may be present in significant amount in liver disease after the administration of large doses of galactose, as in the galactose tolerance test (analogous to the glucose tolerance test) for liver function. Lactose and galactose may be differentiated from other reducing sugars which may be present in the urine by means of the mucic acid test. For a description of the mucic acid test, see the experiments on lactose above. To differentiate galactose, use Tollens' reaction (see the section on pentose above). The red solution given by galactose shows no absorption bands. Galactose is fermentable very slowly or not at all by ordinary bakers' yeast.

FRUCTOSE

The occurrence of fructose in the urine is relatively rare. In essential fructosuria small amounts of fructose are constantly excreted regardless of the fructose content of the diet, although it is curious that, if the diet is carbohydrate-free, the excretion of fructose ceases. No satisfactory explanation has as yet been offered for the condition of essential fructosuria, which is apparently a harmless metabolic abnormality. In diabetes mellitus, fructose may at times be excreted along with glucose (never by itself); the significance of this is not known, although it may be recalled that fructose diphosphate is an intermediate in carbohydrate metabolism (see Chapter 33).

TESTS FOR FRUCTOSE

1. *Borchardt's Reaction.* To about 5 ml. of urine in a test tube add an equal volume of 25 per cent hydrochloric acid and a few crystals of resorcinol. Heat to boiling and after the production of a red color, cool the tube under running water and transfer to an evaporating dish or beaker. Make the mixture slightly alkaline with solid potassium hydroxide, return it to a test tube, add 2 to 3 ml. of ethyl acetate, and shake the tube vigorously. In the presence of fructose the ethyl acetate is colored yellow.

The only urinary constituents which interfere with the test are nitrites and indican, and these interfere only when they are simultaneously present. Under these conditions, the urine should be acidified with acetic acid and heated to boiling for one minute to remove the nitrites. In case the indican content is very large, it will impart a blue color to the ethyl acetate, thus masking the yellow color due to fructose. When such urines are to be examined, the indican should first be removed by Obermayer's test (see p. 804). The chloroform should then be discarded, the acid-urine mixture diluted with one-third its volume of water, and the test applied as described above. The urine of patients who have ingested santonin or rhubarb responds to the test. The test will serve to detect fructose when present in a dilution of 1:2000—i.e., 0.05 per cent.

2. *Resorcinol-Hydrochloric Acid Reaction* (*Selivanoff*). To 5 ml. of Selivanoff's reagent[31] in a test tube add a few drops of the urine under examination and heat the mixture to boiling, or place in a boiling water bath. The presence of fructose is indicated by the production of a red color which may or may not lead ultimately to the separation of a red precipitate. The latter if formed may be filtered off and dissolved in alcohol to which it will impart a striking red color.

If the boiling be prolonged, a similar reaction may be obtained with urine containing glucose. The precautions necessary for a positive test for fructose are as follows: The concentration of the hydrochloric acid must not be more than 12 per cent; the reaction (red color) and the precipitate must be observed after not more than 20–30 seconds of boiling; glucose must not be present in amounts exceeding 2 per cent; the precipitate must be soluble in alcohol with a bright red color.

3. *Aminoguanidine Test* (*Tauber*). See p. 73.

4. *Phenylhydrazine Test*. Make the test according to directions under "Glucose," Exp. 1, p. 824. With methylphenylhydrazine fructose gives crystals differing from those given by glucose in their rate of formation (p. 73).

5. *Polariscopic Examination*. A simple polariscopic examination, when taken in connection with other ordinary tests, will furnish the requisite data regarding the presence of fructose, provided fructose is not accompanied by other levorotatory substances, such as conjugate glucuronates and β-hydroxybutyric acid.

ARSENIC

When any soluble form of arsenic is introduced into the body in any way, it is quickly absorbed and distributed by the blood and lymph. The absorption is influenced by the quantity and character of food in the stomach, and the activity of the circulation of the part in contact with the poison. Some of the absorbed arsenic may be returned to the alimentary canal by way of the bile and gastrointestinal mucous membrane. After absorption it may be deposited in the liver, kidneys, brain, bone, muscles, and walls of the stomach and intestines. It is eliminated in all of the excretions, but chiefly by the kidneys and through the feces. It does not appear very promptly in the urine but continues to be excreted in the urine over a long period of time, in some cases for several months. Testing for arsenic has become of increasing importance because of the widespread use of arsenicals in chemotherapy. Many instances of clinical manifestations of arsenical poisoning have been reported.

DETECTION AND ESTIMATION OF ARSENIC

1. Gutzeit Method:[32] Principle. The presence of arsenic is revealed by the brown stain produced on mercuric bromide paper when arsine is liberated from a test solution by the action of nascent hydrogen.

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[31] See Appendix.

[32] Throughout this determination care must be taken to use clean, arsenic-free reagents

Procedure. Prepare a generator as shown in Fig. 229 using a 50-ml. wide-mouth bottle fitted with a perforated rubber (As-free) stopper. In the central chamber (about 1 cm. wide and 7 cm. long) place loosely packed glass wool or cotton moistened with 10 per cent lead acetate solution. The exit tube should have an internal diameter of about 3 mm. Into this tube insert a strip of mercuric bromide test paper.[33]

To prepare the arsine stain place 5 ml. of 15 per cent potassium iodide, 5 ml. of acid stannous chloride solution (1.6 g. $SnCl_2$ dissolved in 100 ml. 10 per cent HCl), 2 ml. of the test solution, and 30 ml. of water in a flask. Add 1.5 g. granulated As-free zinc and stopper immediately with the exit tube arrangement. Immerse the generator bottle in water at 25° C. for 1 hour. Remove the test strip and compare the color and length of stain with strips similarly prepared from standard arsenic solutions.

Depending upon the nature of the material under examination the test solutions may be prepared by direct solution or by digestion (tissues, foods, urine) with As-free concentrated sulfuric acid as in the Kjeldahl nitrogen determination (see p. 874). To prepare the standard arsenic solutions dissolve 100 mg. arsenic trioxide powder in 5 ml. of 20 per cent NaOH, neutralize with dilute H_2SO_4, add 10 ml. excess acid and make up to 1000 ml. Prepare dilutions of this stock solution with dilute H_2SO_4 so that 2 ml. contain 1, 2, 3, 4 etc. μg. As_2O_3. These amounts will yield a series of stains of graduated length and intensity.

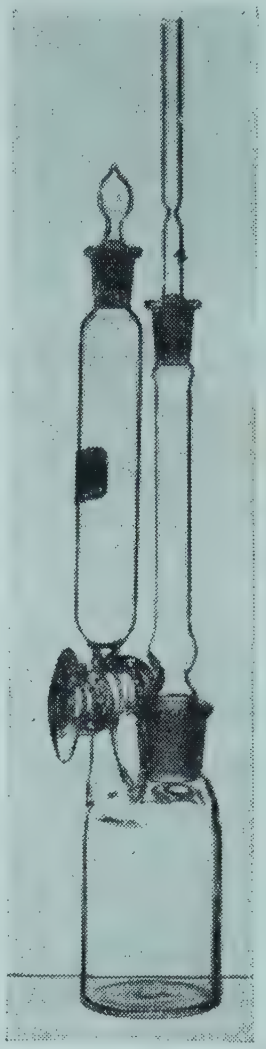

FIG. 229. GUTZEIT ARSENIC APPARATUS.
Courtesy, A. H. Thomas Company, Philadelphia.

2. ***Reinsch's Test.*** This test is very much simpler, but not so delicate. It has the advantage of application in the presence of organic matter. The test is performed as follows: The urine, acidified with one-fifth its volume of pure hydrochloric acid, is placed in a beaker. A piece of bright copper foil free from arsenic is then introduced, and the urine is heated almost to the boiling point. It is then set aside for six to eight hours. The arsenic is deposited on the copper foil, giving it a bluish-gray color. The foil is then removed, washed successively in pure water, alcohol, ether, and dried without heat. The foil is then rolled into a scroll and inserted into a 3-mm.-bore glass tube 4 inches long, about 1 inch from the end. The tube is then held in the Bunsen flame at an angle of 20 to 25 degrees, heat being applied where the copper foil is situated. The arsenic volatilizes and is oxidized, and deposits as octahedral crystals of arsenic trioxide on the cooler part of the tube. The crystals can readily be recognized by the microscope and sometimes with a simple magnifying lens.

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and glassware. A blank determination should be run on the reagents in each generator used in a series. For the precise quantitative determination of arsenic by the Gutzeit method consult *Methods of Analysis of the Association of Official Agricultural Chemists*, 7th ed., 1950, p. 369. Osterberg and Green (*J. Biol. Chem.*, **155**, 513 (1944)) have suggested a rapid method for the microdetermination of arsenic in which the arsenic is converted into arsine by electrolysis.

[33] Test paper strips are prepared as follows: Saturate filter paper with 5 per cent solution of mercuric bromide in alcohol allowing the paper to dry spontaneously while suspended in air. Cut into strips 2.5 mm. × 12 cm. and store in a brown glass bottle.

MERCURY

The rapidity of absorption of mercury depends upon a number of conditions such as mode of administration, the nature of the compound and its physical state, the state and condition of the stomach and intestines, the quantity and quality of the food in the stomach, and the state of the circulation of the portal of entrance. There is no definite knowledge as to the form in which it is absorbed. Elimination depends upon the state of the excretory organs. It is eliminated in all the excretions of the body—urine, feces, saliva, sweat, tears, and milk. Elimination begins about two hours after introduction. Depending upon the amount introduced and absorbed and the extent of renal injury, the time required for its complete elimination varies from 24 hours to many weeks.

TESTS FOR MERCURY

1. *Reinsch's Test.* The procedure is carried out in the same manner as for arsenic (see above). A piece of arsenic-free copper foil is introduced into the urine acidified with one-fifth its volume of pure hydrochloric acid. The urine is, however, not heated to boiling, but warmed to 50° C. or 60° C. and set aside for 12 or preferably 24 hours. Metallic mercury is deposited on the foil as a bright lustrous mirror. The foil is then washed with pure water, alcohol, ether, and dried without heat, rolled into a scroll, inserted into a glass tube and heated in the same manner as under "Arsenic." The mercury is deposited in the metallic state in the form of globules readily distinguishable with the microscope.

2. *Amalgamation Test.* A more rapid method than the above is by amalgamation with zinc. Add 5 g. of zinc dust to the urine and heat for 15 minutes, stirring continuously. Allow the amalgamated zinc to settle and decant the urine. Then wash by decantation several times with pure water, then with alcohol, and finally with ether and dry in air. Now introduce the dry zinc into a narrow dry glass tube sealed at one end. With the Bunsen burner soften the tube about 2 inches above the zinc and constrict the tube by pulling the ends apart. Introduce a small bit of glass wool or asbestos sufficient to support a small piece of iodine. Introduce the iodine supported by the asbestos at the constriction. Apply heat to the zinc amalgam, and then gently to the region holding the iodine to gently volatilize it, and immediately reapply heat to the zinc. The mercury volatilizes and, meeting the iodine vapor, unites with it and is deposited as the red iodide of mercury.

LEAD

Lead may be found to the extent of 0.05 mg. or so per liter in the urine of healthy individuals and may be estimated by the method of Fairhall.[34] It is increased in lead poisoning.

INOSITOL

Inositol, $C_6H_6(OH)_6$, occasionally occurs in the urine in albuminuria, diabetes mellitus, and diabetes insipidus. It is claimed also that copious water drinking causes this substance to appear in the urine. For further discussion, see Chap. 35.

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[34] Fairhall: *J. Biol. Chem.*, **60**, 485 (1924); Aub, Fairhall, Minot, and Reznikoff: *Medicine*, **4**, 1 (1925); Millet: *J. Biol. Chem.*, **83**, 265 (1929). See also Chapters 23 and 31.

TEST FOR INOSITOL

1. *Detection of Inositol* (*Scherer*). Acidify the urine with concentrated nitric acid and evaporate nearly to dryness. Add a few drops of ammonium hydroxide and a little calcium chloride solution to the moist residue and evaporate the mixture to dryness. In the presence of inositol (0.001 g.) a bright red color is obtained.

For a more satisfactory test, which, however, is more time-consuming, see Salkowski's[35] modification of Scherer's test.

FAT

When fat finds its way into the urine through a lesion which brings some portion of the urinary passages into communication with the lymphatic system, a condition known as *chyluria* is established. The turbid or milky appearance of such urine is due to its content of chyle. This disease is encountered most frequently in tropical countries, but is not entirely unknown in more temperate climates. Albumin is a constant constituent of the urine in chyluria. Upon shaking a chylous urine with ether, the fat is dissolved by the ether and the urine becomes less turbid or entirely clear. Alimentary *lipuria* may occur following the ingestion of a large amount of fat.

MELANINS

These pigments never occur normally in the urine, but are present under certain pathological conditions, their presence being especially associated with melanotic tumors. Ordinarily the freshly passed urine is clear, but upon exposure to the air the color deepens and may at last be very dark brown or black in color. The pigment is probably present in the form of a chromogen or melanogen, and upon coming into contact with the air oxidation occurs, causing the transformation of the melanogen into melanin and consequently the darkening of the urine.

It is claimed that melanuria is proof of the formation of a visceral melanotic growth. In many instances, without doubt, urines rich in indican have been wrongly taken as diagnostic proof of melanuria. The pigment melanin is sometimes mistaken for indigo and melanogen for indican. It is comparatively easy to differentiate between indigo and melanin through the solubility of the former in chloroform.

In rare cases melanin is found in urinary sediment in the form of fine amorphous granules.

TESTS FOR MELANIN

1. *Ferric Chloride Reaction* (*von Jaksch-Pollak*). Add a few drops of ferric chloride solution to 10 ml. of urine in a test tube and note the formation of a gray color. Upon the further addition of the chloride a dark precipitate forms, consisting of phosphates and adhering melanin. An excess of ferric chloride causes the precipitate to dissolve.

This is the most satisfactory test for the identification of melanin in the urine.

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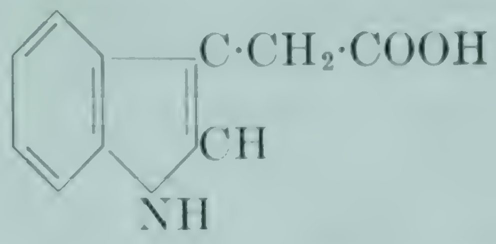
[35] Salkowski: *Z. physiol. Chem.*, **69**, 478 (1910).

2. *Bromine Test* (*Zeller*). To 50 ml. of urine in a small beaker add an equal volume of bromine water. In the presence of melanin a yellow precipitate will form and will gradually darken in color, ultimately becoming black.

UROROSEIN

Urorosein is a urinary pigment which does not occur preformed in the urine, but is present in the form of a chromogen, which is transformed into the pigment upon treatment with a mineral acid. Herter showed this chromogen to be indoleacetic acid,



Indoleacetic acid may be found in urine free or as a compound with glycine. Normal urine responds to the urorosein reaction (see below) if nitrites are present. Pathologically, a positive urorosein reaction is obtained in a variety of diseases, such as pulmonary tuberculosis, typhoid fever, nephritis, stomach disorders, and pellagra. The reaction in the urine of pellagra patients has attracted considerable interest because of its possible metabolic and diagnostic significance.[36]

TEST FOR UROROSEIN

***Nitrite-Hydrochloric Acid Test* (*Urorosein Reaction*). To 10 ml. of urine in a test tube add 2 ml. of concentrated hydrochloric acid and a few drops of a 1 per cent solution of potassium nitrite. A rose-red color indicates urorosein. The chromogen (indoleacetic acid) has been changed to urorosein by oxidation.**

PORPHYRINS[37]

Coproporphyrin and uroporphyrin are present in small amount in normal urine. Both are reddish pigments which may be increased in amount in pathological urine or after administration of quinine, tetronal, trional, or sulfonal.

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[36] Watson: *Proc. Soc. Exptl. Biol. Med.*, **41**, 591 (1939); Watson and Layne: *Ann. Int. Med.*, **19**, 183 (1943).

[37] Dobriner, and Rhoads: *Physiol. Revs.*, **20**, 416 (1940).

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30

Urine: Sediments and Calculi

The sedimentary constituents of the urine may be divided into two classes, viz., organized and unorganized. The sediment is collected by centrifuging the urine at low speed or by allowing it to stand for some time in a conical vessel.

I. UNORGANIZED SEDIMENTS

The more common unorganized sediments consist of ammonium magnesium phosphate ("triple phosphate"), calcium oxalate, calcium phosphate, uric acid, and sodium and ammonium urates. Less commonly observed are calcium carbonate, calcium sulfate, magnesium phosphate, cystine, leucine, tyrosine, hippuric acid, bilirubin, indigo, xanthine, and melanin.

The separation as sediments depends upon the degree of saturation of the urine for these substances, which in turn is influenced by the reaction of the urine. Uric acid most commonly separates out from strongly acid urines, sodium urate from less acid urines. Calcium phosphate comes out most commonly in urines more alkaline than pH 6, while calcium oxalate is found in acid, alkaline, and neutral urines. All of these substances are found as sediments in normal urines, and the majority of normal urines show one or more of these separating out on standing. Ammonium magnesium phosphate, ammonium urate, and calcium carbonate precipitate from urines which have undergone ammoniacal fermentation due to an infection in the urinary tract, and which are hence alkaline in reaction. Maslow studied the sediments forming on long standing in carefully preserved specimens of the urines of normal young men. Sediments were found in 93 per cent of the urines. Uric acid was found in 17 per cent of cases at an average pH of 5.5 and sodium urate in a similar number of cases at an average pH of 5.8. Calcium phosphate was found in 46 per cent of cases with an average pH of 6.2 and calcium oxalate was found in 71 per cent of cases and at all reactions.

Ammonium Magnesium Phosphate ("Triple Phosphate"). Crystals of "triple phosphate" are a characteristic constituent of the sediment when alkaline fermentation of the urine has taken place either before or after being voided. They may even be detected in neutral or slightly acid urine provided the ammonium salts are present in large enough quantity. This substance may occur in the sediment in two forms, viz., prisms and the feathery type. The prismatic form of crystals is the one most commonly observed in the sediment; the feathery form predominates when the urine is made ammoniacal with ammonia (see Fig. 226).

The sediment of the urine in such disorders as are accompanied by a retention of urine in the lower urinary tract contains "triple phosphate" crystals as a characteristic constituent. The crystals are frequently abundant in the sediment during paraplegia, chronic cystitis, enlarged prostate, and chronic pyelitis.

Calcium Oxalate. Calcium oxalate is found in the urine in the form of at least two distinct types of crystals, viz., the dumbbell and "octahedral" types[1] (Fig. 230). Either form may occur in the sediment of neutral, alkaline, or acid urine, but both forms are found most frequently in urine having an acid reaction. Occasionally, in alkaline urine, the octahedral form is confounded with "triple phosphate" crystals. They may be differentiated from the phosphate crystals by the fact that they are insoluble in acetic acid.

The presence of calcium oxalate in the urine is not of itself a sign of any abnormality, since it is a constituent of normal urine. However variations from the normal occur in certain pathological conditions, e.g. parathyroid derangement, urinary calculi, etc.

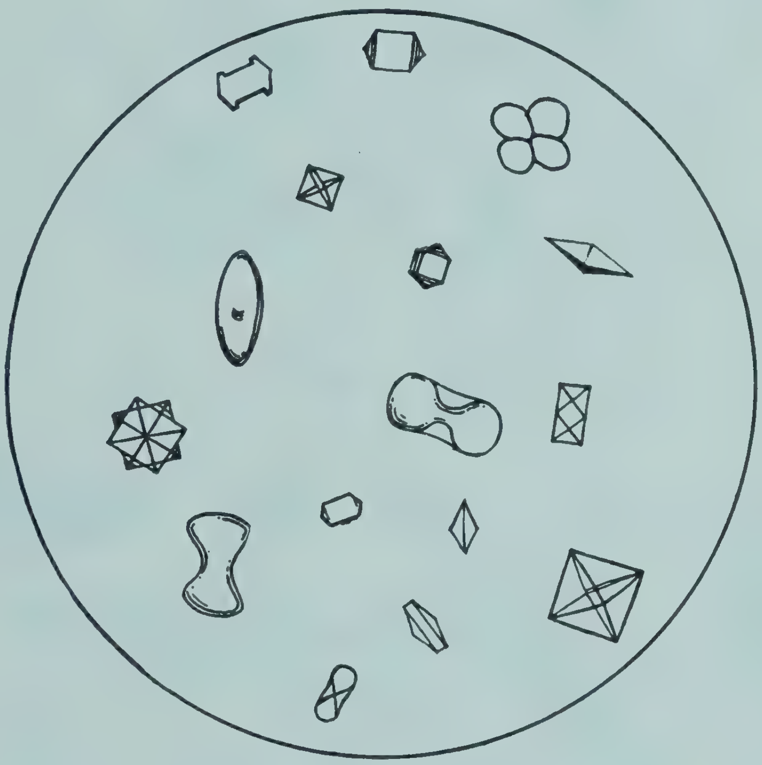

FIG. 230. CALCIUM OXALATE (OGDEN).

Calcium Carbonate. Calcium carbonate crystals form a typical constituent of the urine of herbivorous animals. They occur less frequently in human urine. The reaction of urine containing these crystals is nearly always alkaline, although they may occur in neutral or in slightly acid urine. It generally crystallizes in the form of granules, spherules, or dumbbells (Fig. 231). The crystals of calcium carbonate may be differentiated from calcium oxalate by the fact that they dissolve in acetic acid with the evolution of carbon dioxide gas.

Calcium Phosphate (Stellar Phosphate). Calcium phosphate may occur in the urine in three forms, viz., amorphous, granular, or crystalline. The crystals of calcium phosphate are ordinarily pointed, wedge-shaped formations which may occur as individual crystals or grouped together in more or less regularly formed rosettes (see Fig. 64). Acid sodium urate crystals (Fig. 233) are often mistaken for crystals of calcium phosphate. We may differentiate between these two crystalline forms by the fact that acetic acid will readily dissolve the phosphate, whereas the urate is much less soluble and when it is finally brought into solution and recrystallized, one is frequently enabled to identify uric acid crystals which have been formed from the acid urate solution. The clinical significance of the occurrence of calcium phosphate crystals in the urinary sediment is similar to that of "triple phosphate" (see p. 854).

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[1] The so-called octahedral type is strictly speaking a flat, tetragonal dipyramid identical with the mineral weddelite, whereas the dumbbell-shaped spherulite aggregates correspond to whewellite (Phillipsborn: *Arztliche Forsch.*, **9**, 391 (1953)).

Calcium Sulfate. Crystals of calcium sulfate are of quite rare occurrence in the sediment of urine. Their presence seems to be limited in general to urines which are of a decided acid reaction. Ordinarily it crystallizes in the form of long, thin, colorless needles or prisms (Fig. 225) which may be mistaken for calcium phosphate crystals. There need be no confusion in this respect, however, since the sulfate crystals are insoluble in acetic acid, which reagent readily dissolves the phosphate. As far as is known, their occurrence as a constituent of urinary sediment is of very little clinical significance.

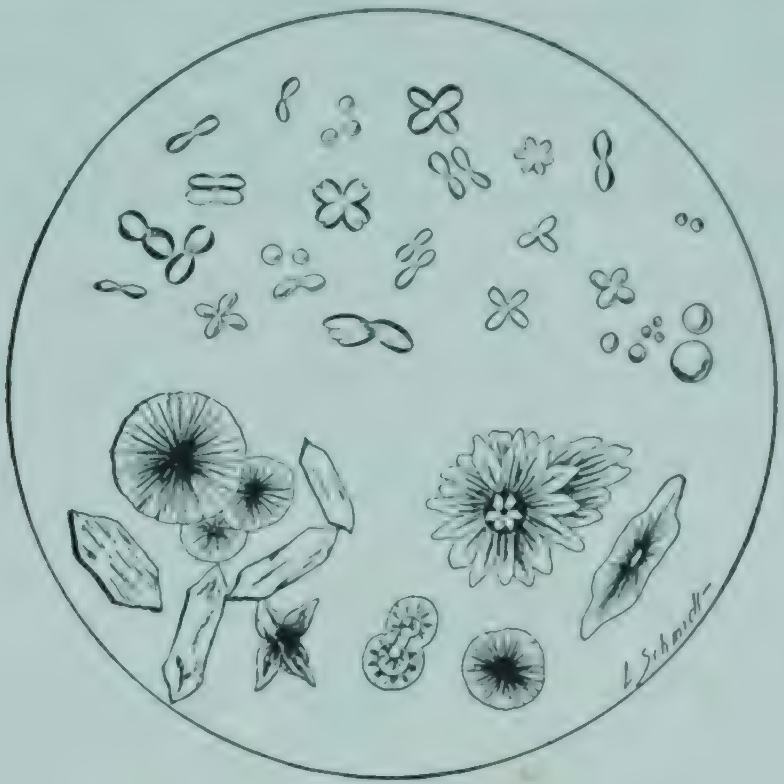


FIG. 231. CALCIUM CARBONATE.

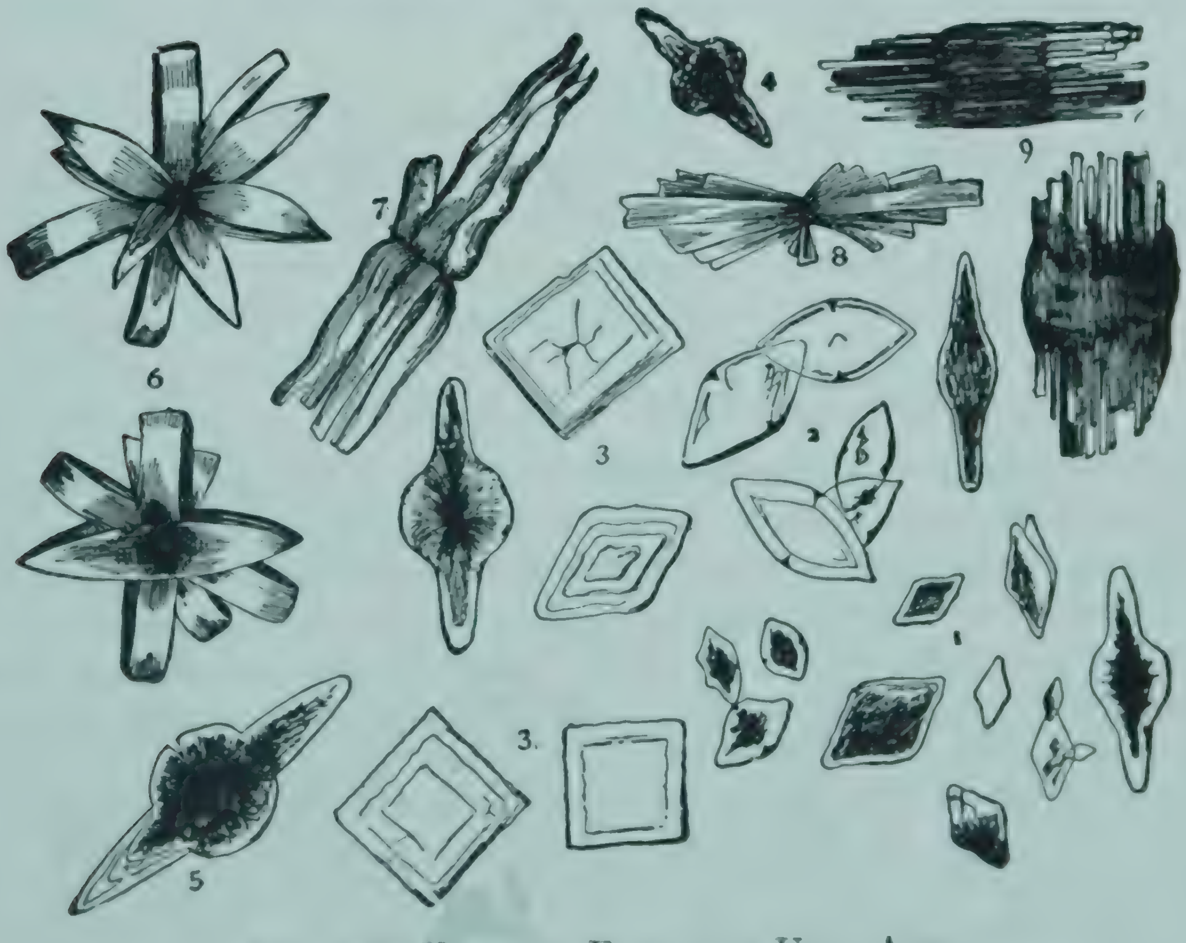


FIG. 232. VARIOUS FORMS OF URIC ACID.

1, Rhombic plates; *2*, whetstone forms; *3*, *3*, quadrate forms; *4*, *5*, prolonged into points; *6*, *8*, rosettes; *7*, pointed bundles; *9*, barrel forms precipitated by adding hydrochloric acid to urine.

Uric Acid. Uric acid forms a very common constituent of the sediment of urines which are acid in reaction. It occurs in more varied forms than any of the other crystalline sediments (see Fig. 232), some of the more common varieties of crystals being rhombic prisms, wedges, dumbbells, whetstones, prismatic rosettes, irregular or hexagonal plates, etc. Crystals of pure uric acid are always colorless (see Fig. 220), but the form occurring in urinary sediments is impure and under the microscope appears pigmented, the depth of color varying from yellow to a dark reddish brown according to the size and form of the crystal.

The presence of a considerable uric acid sediment does not, of necessity, indicate a pathological condition or a urine of increased uric acid content, since this substance very often occurs as a sediment in urines whose uric acid content is diminished from the normal merely as a result of changes in reaction, etc. Pathologically, uric acid sediments occur in gout, acute febrile conditions, chronic interstitial nephritis, etc. If the microscopical examination is not conclusive, uric acid may be differentiated from other crystalline urinary sediments from the fact that it is soluble in alkalies, alkali carbonates, boiling glycerol, concentrated sulfuric acid, and in certain organic bases such as ethylamine and piperidine. It also responds to the murexide test, Schiff's reaction, and to Folin's phosphotungstic acid reaction (see p. 797).

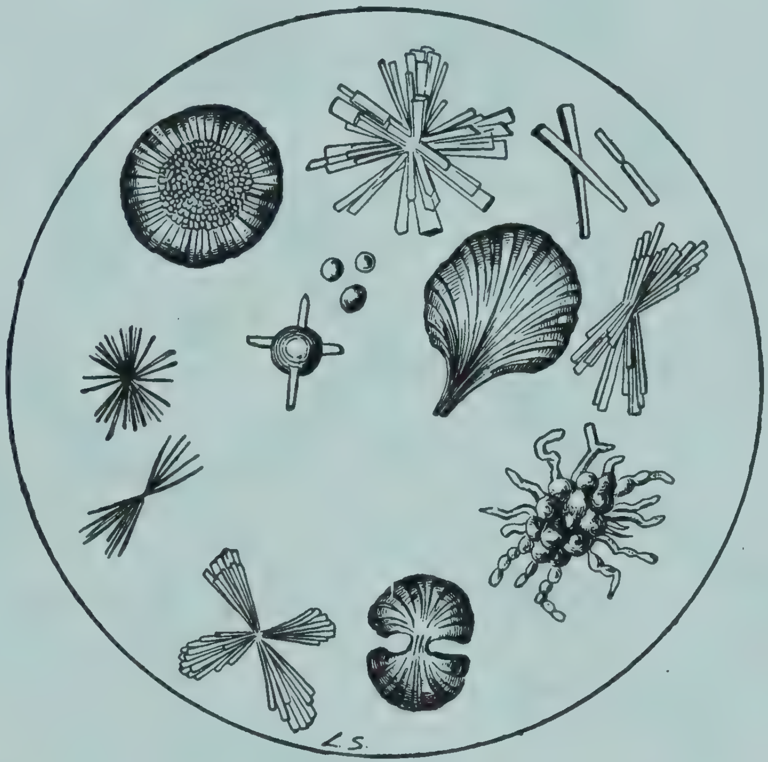
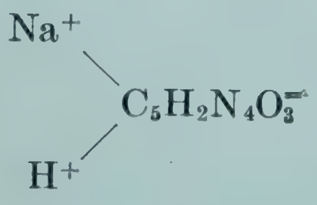
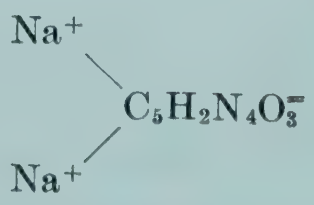


FIG. 233. ACID SODIUM URATE.

Urates. The urate sediment may consist of a mixture of the urates of ammonium, calcium, magnesium, potassium, and sodium. The ammonium urate may occur in neutral, alkaline, or acid urine, whereas the other forms of urates are confined to the sediments of acid urines. Sodium urate occurs in sediments more abundantly than the other urates. There are two sodium urates, the mono- and the di-, which may be expressed thus:


$$Na^+ \quad H^+ \quad C_5H_2N_4O_3^{=}$$

and


$$Na^+ \quad Na^+ \quad C_5H_2N_4O_3^{=}$$

The so-called quadriurate or hemiurate have no existence as chemical units. The urates of calcium, magnesium, and potassium are amorphous in character, whereas the urate of ammonium is crystalline. Sodium urate may be either amorphous or crystalline. When crystalline it forms groups of fan-shaped clusters or colorless, prismatic needles (Fig. 233). Ammonium urate is ordinarily present in the sediment in the burrlike form of the thorn-apple crystal, i.e., yellow or reddish-brown spheres, covered with sharp spicules or prisms (Fig. 234). The urates are all soluble in hydrochloric acid or acetic acid and their acid solutions yield crystals of uric acid upon standing. They also respond to the murexide test. The clinical significance of urate sediments is very similar to that of uric acid. A considerable sediment of amorphous urates does not necessarily indicate a high uric acid content, but ordinarily signifies a concentrated urine having a very strong acidity.

Cystine. Cystine is one of the rarer of the crystalline urinary sediments. It has been claimed that it occurs more often in the urine of men than of women. Cystine crystallizes in the form of thin, colorless, hexagonal plates (see Fig. 45), which are insoluble in water, alcohol, and acetic acid, and soluble in mineral acids, alkalies, and especially in ammonia. Cystine may be identified by burning it upon platinum foil, under which condition it does not melt but yields a bluish-green flame. For the preparation of cystine, see p. 140.

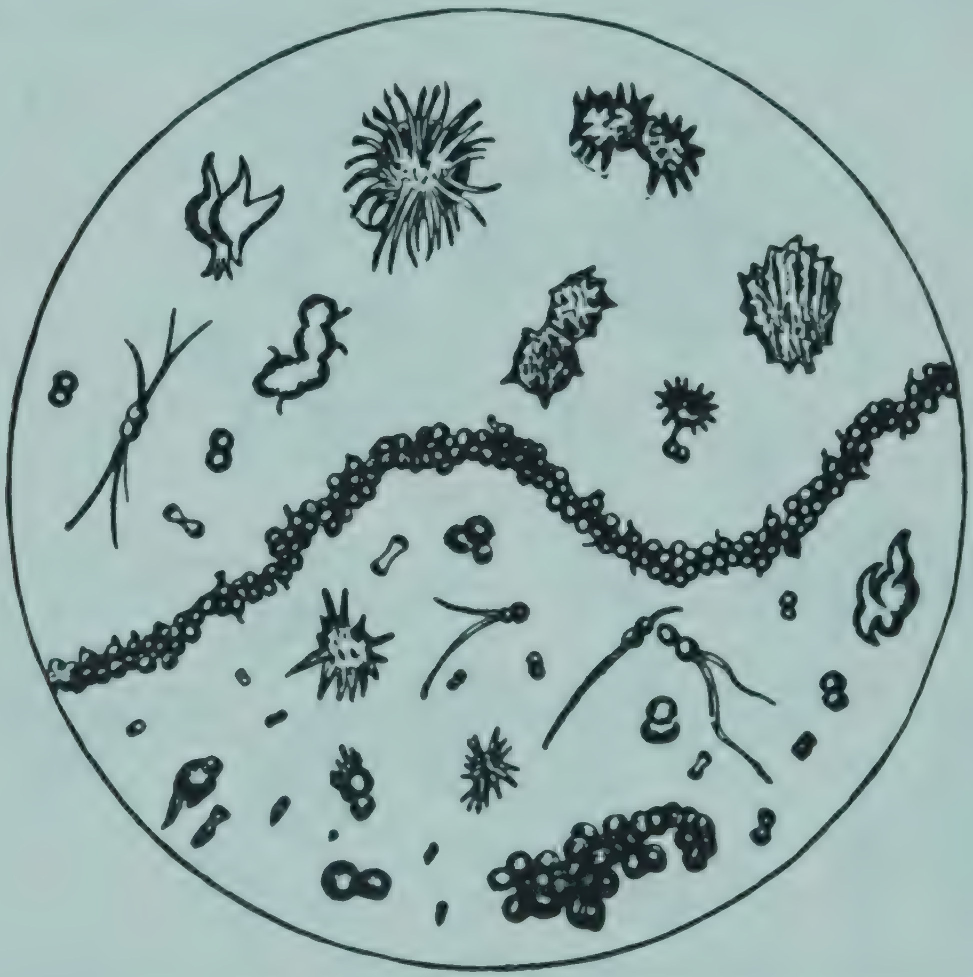


FIG. 234. AMMONIUM URATES, SHOWING SPHERULES AND THORN-APPLE-SHAPED CRYSTALS. (AFTER PEYER.)

Cholesterol. Cholesterol crystals have been but rarely detected in urinary sediments. When present they probably arise from a pathological condition of some portion of the urinary tract. Crystals of cholesterol have been found in the sediment in cystitis, pyelitis, chyluria, and nephritis. Ordinarily they occur as large regular and irregular colorless, transparent plates, some of which possess notched corners (see Fig. 101). Frequently, instead of occurring in the sediment, cholesterol is found in a film on the surface of the urine.

Hippuric Acid. This is one of the rare sediments of human urine. It deposits under conditions similar to those which govern the formation of uric acid sediments. The crystals, which are colorless needles or prisms (see Fig. 223) when pure, are invariably pigmented in a manner similar to the uric acid crystals when observed in urinary sediment and because of this fact are frequently confounded with the rarer forms of uric acid. Hippuric acid may be differentiated from uric acid from the fact that it does not respond to the murexide test and is much more soluble in water and in ether. The detection of crystals of hippuric acid in the urine has very little clinical significance, since its presence in the sediment depends in most instances very greatly upon the nature of the diet. It is particularly prone to occur in the sediment after the ingestion of certain fruits as well as after the ingestion of benzoic acid (see p. 804).

Leucine and Tyrosine. Leucine and tyrosine have frequently been detected in the urine, either in solution or as a sediment. Neither of them occurs in the urine ordinarily except in association with the others, i.e., whenever leucine is detected it is more than probable that tyrosine accompanies it. They have been found pathologically in the urine in acute yellow atrophy of the liver, in acute phosphorus poisoning, in cirrhosis of the liver, in severe cases of typhoid fever and smallpox, and in leukemia. In urinary sediments leucine ordinarily crystallizes in characteristic spherical masses which show both radial and concentric striations and are highly refractive (Fig. 235). Some investigators claim that these crystals which are ordinarily called leucine are, in reality, generally urates. For the crystalline form of pure leucine obtained as a decomposition product of protein, see Fig. 42. Tyrosine crystallizes in urinary sediments in the well-known sheaf or tuft formation (Fig. 44). For other tests on leucine and tyrosine, see pp. 135 and 138.

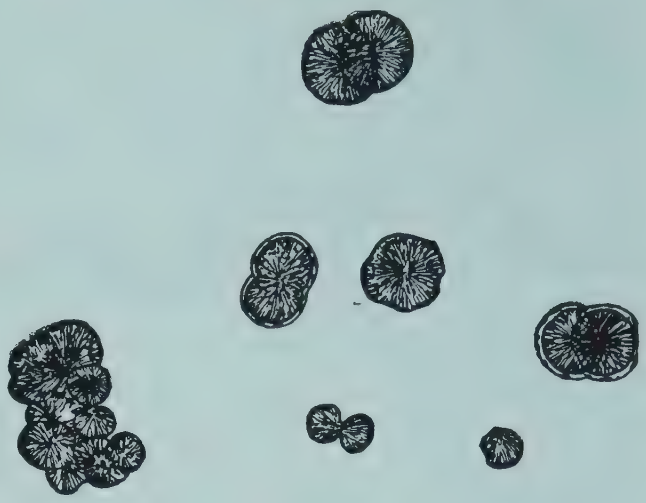


FIG. 235. CRYSTALS OF IMPURE LEUCINE (OGDEN).

Bilirubin. Bilirubin crystallizes in the form of tufts of small needles or in the form of small plates which are ordinarily yellowish red in color (Fig. 100). Pathologically, typical crystals of bilirubin have been found in the urinary sediment in jaundice, acute yellow atrophy of the liver, carcinoma of the liver, cirrhosis of the liver, and in phosphorus poisoning, typhoid fever, and scarlatina.

Magnesium Phosphate. Magnesium phosphate crystals occur rather infrequently in the sediment of urine which is neutral, alkaline, or feebly acid in reaction. It ordinarily crystallizes in elongated, highly refractive, rhombic plates which are soluble in acetic acid.

Indigo. Indigo crystals are frequently found in urine which has undergone alkaline fermentation. They result from the breaking down of indoxyl sulfates or indoxylglycuronates. Ordinarily indigo deposits as dark blue stellate needles or occurs as amorphous particles or broken fragments. These crystalline or amorphous forms may occur in the sediment or may form a blue film on the surface of the urine. Indigo crystals generally occur in urine which is alkaline in reaction, but they have been detected in acid urine.

Xanthine. Xanthine is a constituent of normal urine but is found in the sediment in crystalline form very infrequently, and then only in pathological urine. When present in the sediment xanthine generally occurs in the form of whetstone-shaped crystals somewhat similar in form to the whetstone variety of uric acid crystal. They may be differentiated from uric acid by the great ease with which they may be brought into solution in dilute ammonia and on applying heat. Xanthine may also form urinary calculi. The significance of xanthine in urinary sediment is not well understood.

Melanin. Melanin is an extremely rare constituent of urinary sediments. Ordinarily in melanuria the melanin remains in solution; if it separates it is generally held in suspension as fine amorphous granules.

Sulfonamides. After administration of the sulfonamides (see p. 657 for discussion of chemical nature) crystalline deposits of the free drug or of its acetylated derivative may be found in the urine. Sulfonamide crystals may be recognized by their characteristic appearance (Fig. 236). If the crystals form in the renal tubules during the formation of urine, kidney damage accompanied by hematuria may result. Since the compounds producing the crystals are much more soluble in the form of their alkali salts, the maintenance of an alkaline urine during sulfonamide therapy will prevent the deposition of the crystals.

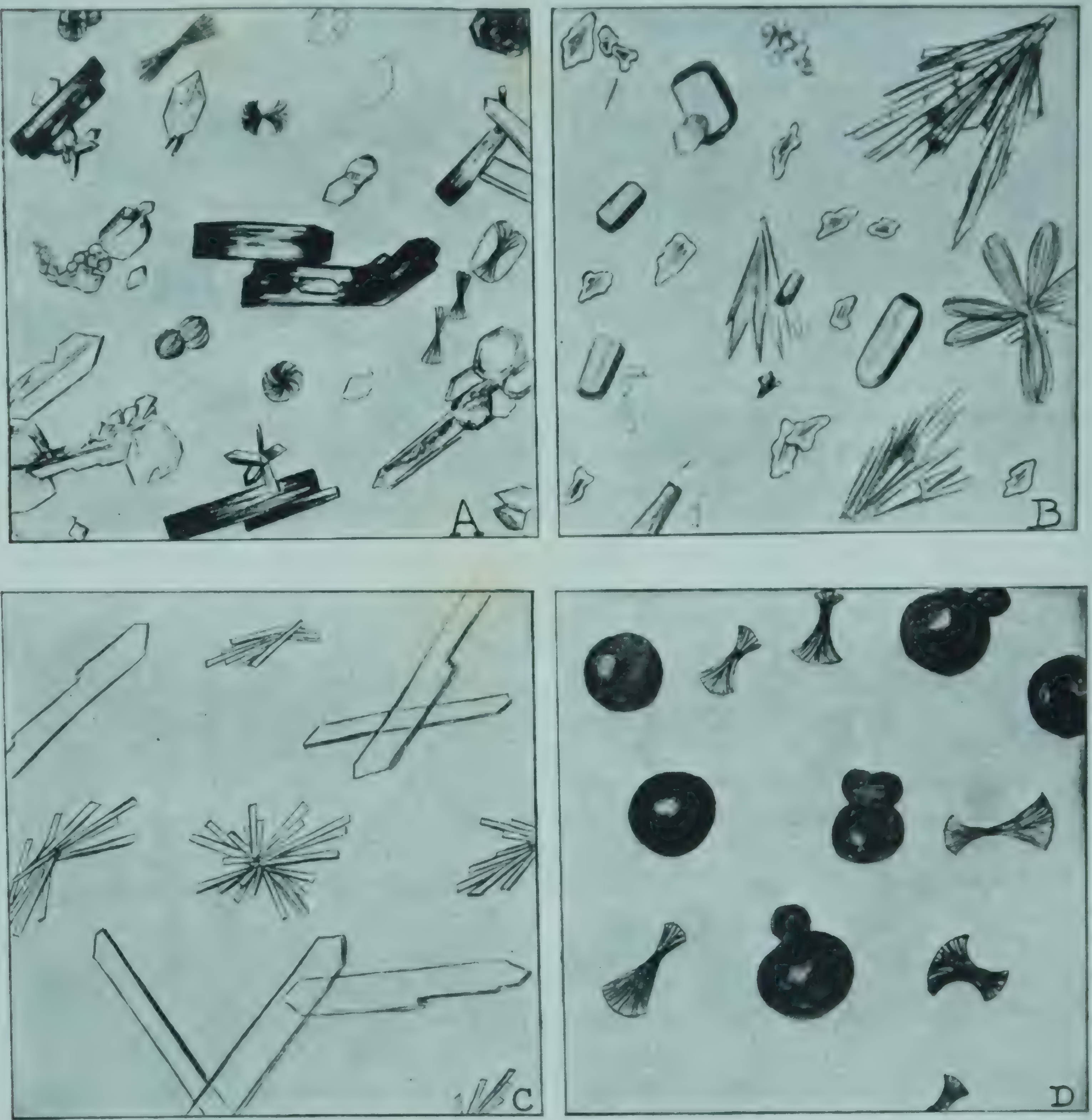


FIG. 236. TYPES OF URINARY CRYSTALS OF THE SULFONAMIDE COMPOUNDS. *A*, Sulfathiazole; *B*, sulfapyridine; *C*, sulfanilamide; *D*, sulfadiazine. Courtesy, Kolmer: *Clinical Diagnosis by Laboratory Examinations*, D. Appleton-Century Co.

II. ORGANIZED SEDIMENTS

Among the more important organized sediments are casts of different types, epithelial cells, pus cells, erythrocytes, and microorganisms. Cylindroids, spermatozoa, urethral filaments, tissue debris, animal parasites, fibrin, and foreign substances due to contamination, are also observed. For detailed discussions of these organized sediments see books on clinical diagnosis or clinical pathology.

CALCULI

Urinary calculi, also called concretions or concrements, are solid masses of urinary sediment formed in some part of the urinary tract. They vary in shape and size according to their location, the smaller calculi, termed sand or gravel, in general arising from the kidney or the pelvic portion of the kidney, whereas the large calculi are ordinarily formed in the bladder. The condition in which calculi are formed in the human body is termed *lithiasis*. Very large stones may also form in the kidney. One of these, the so-called staghorn stone, may fill the entire central portion of the kidney. There are two general classes of calculi as regards composition, viz., simple and compound. The simple form is made up of but a single constituent, whereas the compound type contains two or more individual constituents. The structural plan of most calculi consists of an arrangement of concentric rings about a central nucleus, the number of rings frequently being dependent upon the number of individual constituents which enter into the structure of the calculus. However, layers quite different in macroscopical appearance may be almost identical in composition.

In India and China urinary calculi are most frequently found in children. Up to the middle of the nineteenth century this was true for Europe and America also. But with improvement in diet of more recent years, vesical calculus in children has become rare in this country and is now primarily a disease of old age, more than half of persons admitted for operation being between the ages of 50 and 70. Calculi have been found in Egyptian tombs dating as far back as 4800 B.C.

The etiology of stone in man is still obscure. In rats urinary lithiasis can be regularly produced by diets low in vitamin A. It may be secondary to the epithelial degeneration in the urinary bladder which occurs in vitamin-A deficiency. The calculi contain ammonium magnesium phosphate, calcium carbonate, and calcium hydroxide. The greater frequency of lithiasis in earlier times and the prevalence of stone in children in the Orient may very probably be related to dietary deficiency. Whether vitamin A or other dietary deficiency bears any important relationship to the etiology of calculus disease in this country at the present time is uncertain.

Urinary calculi have frequently been noted in patients with a variety of bone disorders. In certain of these, associated with hyperparathyroidism, the calculus formation may be related to the markedly increased excretion of calcium in the urine. Immobilization of the patient may also be a factor.

Stone formation appears often to be secondary to infection in the urinary tract. Clumps of bacteria and epithelial and pus cells may act as foreign-body nuclei for stone formation and the alkaline fermentation frequently associated with infection is favorable to the precipitation of calcium and ammonium magnesium phosphates, which are the most common constituents of secondary calculi. The majority of stones appear, however, to arise in aseptic urines. That stone formation is not commonly due to any metabolic defect is indicated by the fact that most stones are not composed of any one substance but are of a mixed type.

Attempts have been made to account for the greater solubility of uric acid and other substances in the urine than in pure water, on the basis of the presence, in the urine, of protective colloids which hinder precipitation. According to this view stone formation may occur when this colloid is altered or diminished in quantity. In support of this view it is stated that the crystal form of calcium oxalate in calculi is not that noted to form in pure solutions, but is similar to that produced when precipitation occurs in colloidal solutions. It is possible that precipitation of colloidal material with the crystalloids does conduce to the formation of a concrement rather than a mere precipitate of the latter.

According to the investigation of Meyer, however, the growth of a concrement depends solely on the degree of saturation of the urine for the crystalline constituents and such precipitation of the crystalline constituents occurs in just the same way in urine as in pure aqueous salt solutions. In determining the nature of the sediment or calculus formed the pH is a controlling factor. Uric acid tends to precipitate out of normal urines of average composition when the acidity becomes high (in the neighborhood of pH 5). At pH 6 mixed calculi of uric acid, sodium urate, and calcium oxalate and phosphate will tend to form. At pH 7 calcium phosphate calculi would tend to form and between pH 7 and 8 with the urine ammoniacal mixed calculi of calcium phosphate, magnesium ammonium phosphate, and ammonium urate may form which may be firm if precipitation occurs slowly. At pH values above 8 the rapid precipitation would tend to produce soft stones containing calcium carbonate, ammonium magnesium phosphate, and ammonium urate.

Aside from the question of protective colloids, certain of the stone-forming constituents may, under certain conditions, themselves exist in part in colloidal solution. Thus Hammarsten found that lithium and potassium urates gave true solutions at 37°. Sodium and ammonium urates in saturated solution are present partly in colloidal form. At the average chloride concentration of the urine the solubility products of the latter may be increased 300 per cent. Urates and uric acid in the presence of chlorides thus have a strong tendency to form supersaturated solutions.

According to Stillman,[2] out of 510 urinary calculi submitted for routine examination over a period of five to six years at the New York Hospital, about 44 per cent consisted largely of calcium carbonate, calcium phosphate, or "triple phosphate," either alone or in mixture; about 49 per cent were largely calcium oxalate, with some instances of admixture with phosphate and carbonate; and about 6 per cent were largely uric acid. Cystine calculi occurred to the extent of 0.8 per cent. These examinations refer to the principal constituent of the calculus; no attempt was made to isolate a nucleus and analyze it. Ultzmann has reported that in 545 cases of urinary calculus, uric acid and urates formed the nucleus in about 81 per cent of the cases, earthy phosphates in about 9 per cent, calcium oxalate in about 6 per cent, cystine in a little over 1 per cent, and some foreign body in about 3 per cent.

In the chemical examination of urinary calculi the most valuable data are obtained by subjecting each of the concentric layers of the calculus to a separate analysis. Material for examination may be conveniently obtained by sawing the calculus carefully through the nucleus, then separating the various layers, or by scraping off from each layer (without separating the layers) enough powder to conduct the examination as outlined in the scheme (see p. 863).

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[2] Personal communication. See also Prien and Frondel: *J. Urol.*, 57, 949 (1947).

On heating the powder on platinum foil, it	Burns	Without flame.	The powder gives the murexide test.	The powder when treated with KOH gives	No noticeable ammonia reaction.		Uric acid.
					Strong ammonia reaction.		Ammonium urate.
			Does not give murexide test. The powder dissolves in nitric acid without effervescence. The dried yellow residue becomes orange with alkali, beautiful red on warming.				Xanthine.
		With flame.	Flame pale blue, burns a short time. Peculiar sharp odor. The powder dissolves in ammonia, and six-sided plates separate on the spontaneous evaporation of the ammonia.				Cystine.
			Flame yellow, pale, continuous. Odor of resin or shellac on burning. Powder soluble in alcohol and ether.				Urostealith.
			Flame yellow, continuous. Odor of burnt feathers. Insoluble in alcohol and ether. Soluble in potassium hydroxide with heat. Precipitated therefrom by acetic acid with generation of hydrogen sulfide.				Fibrin.
	Does not burn.	The powder when treated with HCl	Effervesces.				Calcium carbonate.
			Does not effervesce.	The powder gently heated, then treated with HCl	Effervesces.		Calcium oxalate.
					The powder when moistened with a little KOH	No ammonia or, at least, only traces of ammonia. Powder dissolves in acetic acid or HCl. This solution gives an amorphous precipitate with ammonia.	Bone-earth (magnesium and calcium phosphate).
						Abundant ammonia. The powder dissolves in acetic acid or HCl. This solution gives a crystalline precipitate with ammonia.	"Triple phosphate" (mixed with unknown amount of earthy phosphate).

Butt, Hauser, and Seifter[3] have proposed a therapy for renal lithiasis which embraces a pronounced increase in the urinary protective colloids through the subcutaneous injection of *hyaluronidase* in isotonic sodium chloride solution (see "Connective Tissue," p. 243).

VARIETIES OF CALCULI

Uric Acid and Urate Calculi. Uric acid and urates constitute the nuclei of a large proportion of urinary concretions, but stones which consist chiefly of uric acid or urates are found in less than 1 out of 10 cases. Such stones are always colored, the tint varying from a pale yellow to a brownish red. The surface of such calculi is generally smooth but it may be rough and uneven.

Phosphatic Calculi. Ordinarily these concretions consist principally of "triple phosphate" and other phosphates of the alkaline earths, with very frequent admixtures of urates and oxalates. The surface of such calculi is generally rough but may occasionally be rather smooth. The calculi are somewhat variable in color, exhibiting gray, white, or yellow tints under different conditions. When composed of earthy phosphates the calculi are characterized by their friability.

Calcium Oxalate Calculi. These calculi are quite hard and are rather difficult to crush. They ordinarily occur in two general forms, viz., the small, smooth concretion which is characterized as the hemp-seed calculus, and the medium-sized or large stone possessing an extremely uneven surface, which is generally classed as a mulberry calculus. This roughened surface of the latter form of calculus is due, in many instances, to protruding calcium oxalate crystals of the octahedral type.

Calcium Carbonate Calculi. Calcium carbonate concretions are quite common in herbivorous animals, but are of exceedingly rare occurrence in man. They are generally small, white, or grayish calculi, spherical in form, and possess a hard, smooth surface. These considerations apply to stones consisting largely if not entirely of calcium carbonate. Mixed calculi which contain smaller but readily demonstrable amounts of calcium carbonate are rather common in man.

Cystine Calculi. Cystine calculi are of very rare occurrence, the incidence being usually less than 1 per cent. Ordinarily they occur as small, smooth, oval, or cylindrical concretions which are white or yellow in color and of a rather soft consistency.

Xanthine Calculi. This form of calculus is somewhat more rare than the cystine type. The color may vary from white to brownish yellow. Very often uric acid and urates are associated with xanthine in this type of calculus. Upon rubbing a xanthine calculus it has the property of assuming a waxlike appearance.

Urostealith Calculi. This form of calculus is extremely rare. Such concretions are composed principally of fat and fatty acid. When moist they are soft and elastic, but when dried they become brittle. Urostealiths are generally light in color.

Fibrin Calculi. Fibrin calculi are produced in the process of blood coagulation within the urinary tract. They frequently occur as nuclei of other forms of calculus. They are rarely found.

Cholesterol Calculi. This is an extremely rare form of calculus somewhat resembling the cystine type.

Indigo Calculi. Indigo calculi are extremely rare.

The scheme proposed by Heller and given on p. 863 will be found of much assistance in the chemical examination of urinary calculi.

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[3] Butt, Hauser, and Seifter: *J. Am. Med. Assoc.*, **150**, 1096 (1952).

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31

Urine: Quantitative Analysis

In analyzing a normal or pathological urine quantitatively for any of its constituents, it is particularly necessary that the complete and exact 24-hour sample be obtained. For directions with regard to the collection and preservation of urine for analysis, see Chapter 27 "General Characteristics of Normal and Pathological Urine." Methods for the determination of the specific gravity of the urine are also there described. Before any urine is taken for analysis its total volume should be measured, using a large graduated cylinder, and this volume is thereafter taken as a basis for the calculations of the daily output of the individual constituents determined.

PREPARATION OF STANDARD ACID AND ALKALI SOLUTIONS

Principle. Many of the quantitative methods used in physiological chemistry are volumetric or titration procedures. For these methods solutions of accurately known strength called standard solutions are needed. Their strength is usually expressed in terms of normality. A normal solution is one which in 1000 ml. contains 1 g. of replaceable hydrogen or its equivalent. Thus, to make 1000 ml. of a normal solution of hydrochloric acid (HCl), we would need 36.5 g. of this acid containing 1 g. of replaceable hydrogen. This we derive from the fact that the atomic weight of Cl is 35.5 and of H is 1, so that the molecular weight of HCl is 36.5, and each 36.5 g. of this acid contains 1 g. of replaceable hydrogen.[1] Sulfuric acid (H_2SO_4) has a molecular weight of $2 + 32 + 64 = 98$, but 98 g. of sulfuric acid contains 2 g. of replaceable hydrogen. Therefore, to prepare a normal solution of this acid, we must use one-half of 98 or 49 g. of sulfuric acid (containing 1 g. of hydrogen) for 1000 ml. of normal solution. Oxalic acid ($H_2C_2O_4 + 2H_2O$) has a molecular weight of $2 + 24 + 64 + 36 = 126$. It also is a dibasic acid so we must use only one-half of 126 or 63 g. of oxalic acid in making a liter of normal solution.

A normal alkali solution is exactly equivalent to a normal acid solution, i.e., 1 liter of the alkali will neutralize 1 liter of the acid. According to the reaction of neutralization, therefore, the 36.5 g. of HCl in a liter of this normal acid will require 40.0 g. of sodium hydroxide to neutralize it, and 1 liter of normal sodium hydroxide must contain 40.0 g. of the alkali.

$$\underset{(36.5)}{HCl} + \underset{(40.0)}{NaOH} \rightarrow \underset{(58.5)}{NaCl} + \underset{(18)}{H_2O}$$

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[1] See table of atomic weights, Appendix.

Having prepared solutions of acid and alkali of definitely known strength, it is then possible to determine the strength of any unknown acid or alkali by finding out much much of these standard solutions is required to neutralize a definite volume of the unknown solution.

In order to tell when the unknown solution has been exactly neutralized, we titrate in the presence of a small amount of one of a class of substances called indicators. An indicator is a substance which undergoes a sharp color change at a particular range of hydrogen-ion concentration, and this color change indicates the end-point of the titration.

When strong acids (as HCl) are being titrated with strong alkalies (as NaOH), almost any one of the common indicators is satisfactory. If weak acids (acetic acid) or weak bases (as ammonia) are being titrated, it is necessary to be very careful in the choice of an indicator as all indicators are not equally satisfactory in these instances.[2] (See p. 39.)

Preparation of 0.1 N Oxalic Acid Solution. **Weigh accurately a watch glass or a piece of glazed paper. Then add to the weights on the balance pan 3.1512 g. With a spatula transfer to the watch glass enough pure oxalic acid in the form of clear crystals to counterbalance exactly the weights in the opposite pan. Transfer completely to a 250-ml. beaker, using a little water for rinsing purposes. Add about 150 ml. of distilled water and stir with a glass rod until dissolved, warming gently if necessary. Transfer every particle of this solution to a clean 500-ml. volumetric flask, rinsing rod and beaker several times with distilled water. Hold under the tap until cooled to room temperature. Then add distilled water until the bottom of the meniscus is level with the mark on the neck of the flask (the lower mark if there are two). Insert a stopper and mix thoroughly by inverting the flask again and again. Transfer to a clean dry bottle. Label. This solution will not keep indefinitely and is to be used only in the standardization of 0.1 N alkali.**

Preparation of 0.1 N Sodium Hydroxide Solution.

(a) PREPARATION OF CONCENTRATED CARBONATE-FREE SODIUM HYDROXIDE SOLUTION. **Shake up about 110 g. of best quality NaOH with 100 ml. of distilled water in a 300-ml. Erlenmeyer flask (pyrex) to make a saturated solution. Stopper and allow to stand for a couple of days or until the sodium carbonate settles to the bottom, leaving a clear solution of NaOH practically free from carbonate.**

(b) PREPARATION OF A STANDARD SODIUM HYDROXIDE SOLUTION. **Measure out 6.3 ml. of the saturated NaOH solution from a buret into a 1-liter flask. Add 750 ml. of distilled water and mix thoroughly. Clean a buret by allowing it to stand filled with cleaning mixture (sodium dichromate and sulfuric acid) for a few minutes or longer if necessary. Empty, rinse several times with tap water, finally with distilled water, and allow to drain. Introduce a few ml. of the NaOH solution, and invert a couple of times to rinse the buret, discarding this NaOH. Repeat this process at least twice more. Then fill the buret with the alkali solution, making sure that the tip contains no air bubbles, and run out solution until the bottom of the meniscus is exactly at 0.**

Into a clean Erlenmeyer flask (150 to 250 ml.) now introduce 25 ml. of 0.1 N oxalic acid solution measured from an accurate, clean pipet, previously rinsed by means of a little of the acid solution drawn up into it. Allow the pipet to drain about 15 seconds against the side of the flask. Add 2 to 3 drops of a 1 per cent alcoholic solution of phenolphthalein.

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[2] For further consideration of indicators see pp. 38 and 378.

Now run in NaOH solution from the buret, rotating the flask. Ten ml. can be added quite rapidly, then add more slowly, and finally drop by drop until the last drop changes the color of the solution permanently throughout to a definite pink. Take the buret reading, estimating as closely as possible to the second decimal place. Repeat the titration until two closely agreeing duplicate readings are obtained, then average the two readings for calculation.

Calculate the strength of the NaOH solution. Divide 25 (the number of ml. of 0.1 N oxalic acid used) by the buret reading to obtain the strength of the NaOH in terms of 0.1 N solution. Then multiply by 0.1 to obtain the normality. For example, if 15.67 ml. were required: 25 ÷ 15.67 = 1.595; 1.595 × 0.1 = 0.1595 N.

(c) PREPARATION OF THE 0.1 N NaOH SOLUTION. Calculate how much of the standard NaOH solution just prepared will be required to make 1 liter of 0.1 N solution. To do this divide 1000 ml. by the strength of the NaOH in terms of 0.1 N solution. Thus in the example cited above: 1000 ÷ 1.595 = 626.9 ml. required. Measure out the exact amount of alkali required (using the buret, pipet, and volumetric flasks) into a 1000-ml. flask. Dilute with distilled water exactly to the mark. Mix very thoroughly and transfer to a clean, dry bottle with a rubber (not glass) stopper. Check the strength of the solution by again titrating 25-ml. portions of oxalic acid solution.[3]

Preparation of 0.1 N Hydrochloric Acid. Concentrated hydrochloric acid is about 12 N or 44 per cent HCl weight in volume. Approximately 0.1 N HCl may, therefore, be prepared by diluting 9 ml. of the concentrated acid to 1 liter in a volumetric flask. This must be standardized by titration with 0.1 N alkali, using alizarin red or methyl red as an indicator.

Or introduce into a 1-liter flask 12 ml. of concentrated HCl and 750 ml. of distilled water. Mix well and titrate 10, 15, or 25 ml. portions of the acid solution with 0.1 N NaOH, using alizarin as an indicator. Dividing the number of ml. of 0.1 N NaOH required by the number of ml. of acid used gives the strength of the HCl in terms of 0.1 N solution. Dividing 1000 by this quotient gives the number of ml. of HCl solution to be measured into a volumetric flask and made up to 1000 ml.

This diluted solution will be 0.1 N HCl. It should be mixed thoroughly and 25-ml. portions of it checked by titration with the 0.1 N NaOH.[4]

Standard acid and alkali solutions are best kept in paraffin-lined bottles. The acid solution is the more permanent of the two; and standard sulfuric acid solutions are more permanent than hydrochloric acid solutions. Alkali solutions must be protected from the carbonic acid of the air, the solution being best drawn over into the buret by means of a siphon tube leading from the top of the buret to the interior of the alkali bottle. The air inlet through the stopper of the bottle should be guarded by a tube containing soda lime.

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[3] If a very high degree of accuracy is desired, the alkali may be checked against pure acid potassium phthalate (mol. wt. 204.139). Dodge: *J. Ind. Eng. Chem.*, 7, 29 (1915); *J. Am. Chem. Soc.*, **42**, 1655 (1920).

[4] The acid solution may be standardized directly in the following manner: Introduce a platinum dish containing very pure sodium bicarbonate or the highest grade anhydrous sodium carbonate into a hot-air oven previously heated to 200° C. Raise the temperature to 270° to 280°, but not above 300° C. Heat for half an hour, allow to cool in a desiccator, but while still a little warm, transfer to a glass-stoppered weighing bottle. Weigh out rapidly 0.1- to 0.2-g. portions of the sodium carbonate, dissolve in about 50 ml. of water in an Erlenmeyer flask, and titrate, using methyl orange as an indicator. One hundred ml. of 0.1 N acid is equivalent to 0.530 g. of dried sodium carbonate.

Preparation of Standard Hydrochloric Acid from a Constant Boiling Solution (Method of Hulett and Bonner): Procedure. **Make up by hydrometer about 200 ml. of HCl of specific gravity about 1.10. Distil off about three-fourths of the liquid and discard. Then collect about 25 ml. of the constant-boiling distillate. Weigh 18.017 g. of distillate using a capillary pipet for final adjustment (or measure out 16.442 ml. at 25° C.). Dilute to 1 liter. This is a 0.1 N solution of HCl. The figures given are for a barometric pressure of 760 mm. At 770 mm. use 18.039 g.; at 750 mm. use 17.996 g.; at 740 mm. use 17.975 g. and at 730 mm. use 17.953 g. The composition of the distillate should not vary more than 1 part in 10,000 from the figures given.**

TITRATABLE ACIDITY OF URINE

Introduction. The titratable acidity of the urine, as determined by the Folin method described here, is expressed in terms of the amount of standard alkali necessary to bring the urine from its original pH to the phenolphthalein end point, around pH 8.5 or 9. In normal urine, the "acid" titrated consists almost entirely of the acid phosphate ion, $H_2PO_4^-$, the reaction being as follows:

$$H_2PO_4^- + OH^- = HPO_4^= + H_2O$$

Small amounts of titratable acidity may be contributed normally by acid organic salts (acid urate, acid oxalate, etc.). In certain conditions, such as severe ketosis, significant amounts of free β-hydroxybutyric acid, for example, may be present in the urine and contribute to the titratable acidity. In general, however, monobasic organic acids are found in the urine largely if not entirely as their salts, and in this form contribute little or nothing to the total titratable acidity. To evaluate the significance of the excretion of organic acids on acid-base balance, they may be determined as such (see "Determination of Organic Acids," p. 871) or the urinary ammonia content determined as an index of base replacement (see p. 888).

The Folin method is theoretically unsound as an exact measure of acid excretion relative to the normal acid-base balance in the body, and cannot be used for such purposes. A more accurate picture is obtained if the urine is titrated back to the pH of the blood (pH 7.4) rather than to pH 9, since it is the difference between the pH of blood and urine which reflects the excretion of acid or base in excess of the amounts normally required for acid-base balance. A method for determining titratable acidity by titrating urine to pH 7.4 against a color standard is described by Henderson and Palmer;[5] a pH meter may also be used. In the Folin method, urines even more alkaline than the blood may show a positive "titratable acidity," the quantitative significance of which is obscure. Despite these limitations, the method is simple and has been widely used, especially for comparative purposes and in conjunction with the determination of urinary ammonia as an aid in estimating the severity of acidosis, as described by Fitz and Van Slyke.[5a]

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[5] Henderson and Palmer: *J. Biol. Chem.*, 17, 305 (1914).

Folin's Method: Principle. The urine is titrated with standard sodium hydroxide solution, using phenolphthalein as an indicator. Potassium oxalate is added to precipitate the calcium which would otherwise interfere with the end point due to the precipitation of calcium phosphate on neutralization of the urine.

Procedure. **Place 25 ml. of urine in a 200-ml. Erlenmeyer flask and add 5 grams of finely pulverized potassium oxalate and 1 to 2 drops of a 1 per cent phenolphthalein solution to the fluid. Shake the mixture vigorously for 1 to 2 minutes and titrate it immediately with 0.1 N sodium hydroxide until a faint but unmistakable pink remains permanent on further shaking. For more accurate results, particularly with deeply colored urine, compare during the titration against a control sample of urine plus oxalate, until there is a distinct difference in color in the titrated sample. The control sample may then be used for a duplicate estimation, titrating it to color match against the first sample. Take the buret reading and calculate the titratable acidity of the urine under examination.**

CALCULATION. If y represents the number of milliliters of 0.1 N sodium hydroxide used and y' represents the volume of urine excreted in 24 hours, the total acidity of the 24-hour urine specimen (x) may be calculated by means of the following proportion:

$$25/y = y'/x \text{ (titratable acidity of 24-hour urine expressed in ml. of 0.1 NaOH)}$$

Interpretation.[6] The titratable acidity of the urine, expressed in ml. of 0.1 N alkali required to neutralize the 24-hour output by the method described, varies ordinarily from 200 to 500 under normal conditions with an average of perhaps 350. It is dependent almost entirely upon the diet, being low on a vegetable (base-forming) diet and high on a diet containing much meat, milk, cheese, rice, whole wheat products, etc. (acid-forming foods). On the administration of 15 g. of sodium bicarbonate it may go down to 100; the ingestion of much acid-forming food may increase it to 600. In fasting it may rise in a few days to 800. Acidities of less than 250 usually indicate a true alkalinity of the urine inasmuch as phenolphthalein changes at a pH significantly more alkaline than that of the blood, as discussed above. Samples of urine collected shortly after a meal may be alkaline due to the so-called "alkaline tide."

Bacterial decomposition of the urea of the urine occurring in the urinary tract will increase the amount of ammonia and decrease the acidity of the urine. The same change usually occurs in urine left in contact with the air. The acidity of the urine is increased in acidosis and cardiorenal and certain other disorders. The acidity of the urine may be somewhat increased by administration of mineral acids, acid phosphates, or ammonium chloride, but it is much more difficult to increase than to decrease this acidity.

[5a] Fitz and Van Slyke: *J. Biol. Chem.*, **30**, 389 (1917); Van Slyke: *J. Biol. Chem.*, **33**, 271 (1918); Barnett: *J. Biol. Chem.*, **33**, 267 (1918).

[6] Under the heading "Interpretation" there will be found, in connection with the various quantitative methods which follow, brief notes as to the possible significance of the results obtained. For further discussion see Chapters 27, 33, and 34. General references will be found listed at the end of the chapter, particularly with reference to clinical aspects.

Determination of Organic Acids (Method of Van Slyke and Palmer):[7] ***Principle.*** Carbonates and phosphates are precipitated and the filtrate titrated with acid from pH 8 to pH 2.7. In the procedure described, the indicators phenolphthalein and tropeolin OO are used to define the pH range; a pH meter may also be used. Variations in the technique are required in the albuminous or high-bicarbonate urines.

Procedure. **Mix 100 ml. of urine with 2 g. of finely powdered calcium hydroxide and stir occasionally for 15 minutes. Filter. Carbonates and phosphates are removed. Transfer 25 ml. of filtrate to a 125- to 150-ml. test tube, add 0.5 ml. of 1 per cent phenolphthalein and 0.2 N HCl from a buret until pink color just disappears. This amount need not be measured. The pH is now about 8. Add 5 ml. of 0.02 per cent tropeolin OO[8] little by little with stirring to prevent precipitation of the dye. Titrate with 0.2 N HCl until the red color matches that of a standard (pH 2.7) made from 0.6 ml. 0.2 N HCl, 5 ml. of tropeolin OO solution and water to make 60 ml. When the end point is nearly reached dilute unknown to 60 ml. before completing the titration.**

To avoid the buffering effect of protein in albuminous urine, acidify with a few drops of concentrated HCl, boil, and filter before proceeding with the addition of calcium hydroxide as described above. Similar acidification and aeration by pouring will sufficiently reduce the CO_2 content of high-bicarbonate urines (pH over 7).

CALCULATION. Subtract from ml. 0.2 N HCl the amount (usually 0.7 ml.) of the acid required to titrate a control tube of water between the same limits. Multiply the difference by 2 to get results in terms of 0.1 N acid (the usual basis) and by 1000/25 to get the value for 1000 ml. of urine.

Interpretation. The titration includes organic acids, creatine, creatinine, and a small amount of amino acids. Normally the excretion for 24 hours corresponds to 400 to 750 ml. of 0.1 N HCl or about 8 ml. per kg. of body weight. In diabetic acidosis values from 20 to 180 ml. per kg. have been observed. In diabetes this titration approximates closely the amount of β-hydroxybutyric and acetoacetic acids in the urine and may be substituted for the determination of these substances for clinical purposes.

HYDROGEN-ION CONCENTRATION OR TRUE ACIDITY

Colorimetric Method: Principle. The reaction of the urine is determined by matching the colors produced when pH indicators are added respectively to the diluted urine and to the standard solutions of known reaction. This method is subject to salt and dilution errors but is sufficiently accurate for most clinical purposes.

Procedure: Preparation of Standard Buffer Solutions. **The Sørensen phosphate buffer standards, together with the Walpole acetate standards, cover satisfactorily the range of pH of urine. The acetate buffers are prepared by mixing 0.1 N acetic acid and 0.1 N sodium acetate in the following proportions:**

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[7] Van Slyke and Palmer: *J. Biol. Chem.*, **41**, 567 (1920). Palmer: *J. Biol. Chem.*, **68**, 245 (1926). According to Widmark and Ljungberg (*Biochem. Z.*, **216**, 1 (1929)) the CaO used in this method carries down from 7 to 10 per cent of the ether-soluble acids of urine and thus gives slightly low results.

[8] In certain urines this indicator fades under acid conditions. Bromophenol blue may be substituted or, preferably, a pH meter may be used.

pH	*0.1 N Acetic Acid*	*0.1 N Sodium Acetate*
	ml.	*ml.*
3.6	185	15
3.8	176	24
4.0	164	36
4.2	147	53
4.4	126	74
4.6	102	98
4.8	80	120
5.0	59	141
5.2	42	158
5.4	29	171
5.6	19	181

The phosphate buffers are prepared by mixing M/15 disodium phosphate and M/15 potassium acid phosphate in the proportions[9] given in the table below.

pH	*M/15* Na_2HPO_4	*M/15* KH_2PO_4
	ml.	*ml.*
5.4	3.0	97.0
5.6	5.0	95.0
5.8	7.8	92.2
6.0	12.0	88.0
6.2	18.5	81.5
6.4	26.5	73.5
6.6	37.5	62.5
6.8	50.0	50.0
7.0	61.1	38.9
7.2	71.5	28.5
7.4	80.4	19.6
7.6	86.8	13.2
7.8	91.4	8.6
8.0	94.5	5.5

If it is desired to use the Clark and Lubs standard buffer mixtures these may be prepared as described on p. 35, *et seq.*

Choice of Indicator. The selection of indicators depends on the pH range. See the table on p. 381. For urine, bromocresol green (pH 4.0 to 5.6), bromocresol purple (pH 5.4 to 7.0) and phenol red (pH 6.6 to 8.2) may be used. Methyl red (pH 4.4 to 6.0) may be substituted for bromocresol green, but is not so stable. Aqueous solutions of 0.04 per cent strength are used. Ten drops (or 0.5 ml.) of these indicators are added to 10-ml. portions of the standard buffer solutions covering the effective pH range of the particular indicator. For preparation of indicator solutions, see p. 39.

Determination. The urine is collected and kept under mineral oil. Into a test tube of the same diameter as those used to contain the indicator stand-

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[9] Interpolated from the data of Sørensen. For the preparation of the M/15 solutions see p. 35.

ards, introduce 8 ml. of recently boiled distilled water and 10 drops (or 0.5 ml.) of a particular indicator solution, say bromocresol purple. To this diluted indicator solution now add a layer of mineral oil and then introduce, without exposure to air, 2 ml. of urine. Stir gently and match with indicator standards in a comparator block. A control tube containing the urine diluted 1:5 should be used in making the comparison, as described in the hydrogen-ion determination of blood (see p. 699).

If the urine is too acid or alkaline to come within the range of the indicator selected, repeat, using a more suitable indicator, until a satisfactory one is found.

CALCULATION. The colorimetric reading made on the diluted urine at room temperature may be approximately corrected to give the actual pH at 38° C., by subtracting 0.2 pH. The latter factor is, however, not constant, Hastings, Sendroy, and Robson warm the diluted urine to 38° C. before reading, and correct only for the dilution by subtracting 0.1 pH. This gives results which are claimed to be within 0.05 pH of the electrometric pH of the undiluted urine at 38° C.

Interpretation. The pH of urine may vary over the extreme range of pH 4.8 to 8.0; normal pH values lie between about 5.5 and 8.0, with a mean value of about 6.0. In most pathological conditions the mean pH is lowered (increased acidity). The factors influencing urinary pH are similar to those influencing the titratable acidity of the urine (diets containing acid- and base-forming foods and the administration of acid or alkali); the two are not necessarily related quantitatively, however. Thus a dilute urine and a concentrated urine may have the same pH, but quite different titratable acidities. The pH may be considered as an *intensity* factor, while the titratable acidity is a *capacity* factor. The determination of urinary pH is of importance clinically largely in relation to the precipitation of insoluble material from the urine and the possible formation of urinary calculi. For example, in acid urine, urates and certain sulfonamides may precipitate out; prophylaxis entails the maintenance of an alkaline urine in which these substances are more soluble. The reverse is true for phosphate calculi, which form in alkaline urine but are soluble in acid urine. Urinary pH may be lowered by the administration of ammonium chloride or raised by sodium bicarbonate.

Electrometric Method. The colorimetric determination of urinary pH has been largely replaced for research purposes by the use of the glass-electrode pH meter (see p. 48). More accurate results are obtained, and color or turbidity of the urine does not influence results.

TOTAL SOLIDS

***Drying Method.* Place 5 ml. of urine in a weighed shallow dish, acidify *very slightly* with acetic acid (1 to 3 drops), and dry it *in vacuo* in the presence of sulfuric acid to constant weight. Calculate the percentage of solids in the urine sample and the total solids for the 24-hour period.**

Interpretation. The average excretion of total solids by a normal adult man is about 70 g. It is largely dependent upon the protein and salts of the diet. It may be decreased in severe nephritis due to impaired excretion, and greatly increased in diabetes with high sugar elimination.

Practically all the methods whose technique includes evaporation at an increased temperature, either under atmospheric conditions or *in vacuo*, are attended with error.

Shackell's method which entails the vacuum desiccation of the frozen sample is extremely satisfactory and should be used where the greatest accuracy is desired.

Long's coefficient (2.6) was at one time used to compute the solids content of urine. The second and third decimal figures of the specific gravity were multiplied by 2.6 to give the number of grams of solid matter in 1 liter of urine. This procedure is now obsolete.

TOTAL NITROGEN

1. Kjeldahl Method:[10] ***Principle.*** The principle of this method is the conversion of the various nitrogenous compounds of the urine into ammonium sulfate by boiling with concentrated sulfuric acid, the subsequent decomposition of the ammonium sulfate by means of a fixed alkali (NaOH), and the collection of the liberated ammonia in an acid of known strength. Finally, this partly neutralized acid solution is titrated with an alkali of known strength and the nitrogen content of the urine under examination computed.

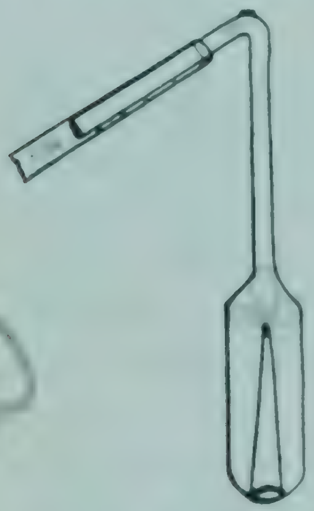

FIG. 237. FOLIN FUME ABSORBER.

Procedure. **Place 5 ml. of urine in a 500-ml. long-necked pyrex-glass Kjeldahl flask, add 20 ml. of concentrated sulfuric acid and about 0.2 g. of copper sulfate and boil the mixture until it becomes light green or almost colorless, and then for about an hour longer. Avoid too strong a flame at this point, to prevent loss of acid. If a suitable hood or fume chamber is not available the sulfuric acid vapors may be carried away by suction. Various devices for this purpose are listed in laboratory supply dealers' catalogues; the Folin fume-absorber (Fig. 237), placed in the mouth of the flask, may also be used.**

When oxidation of the sample is complete, allow the flask to cool and dilute the contents with about 200 ml. of ammonia-free water. Add a little more of a concentrated solution of NaOH than is necessary to neutralize the sulfuric acid[11] **down the side of the flask in such a way that the alkali does not mix with the solution in the flask but forms a layer beneath it, and introduce into the flask a little coarse pumice stone, a scoop of talcum, or a few pieces of granulated zinc,**[12] **to prevent bumping. A small piece of paraffin may be added to lessen the tendency to froth. By means of a safety tube connect the flask with a condenser so arranged that the delivery tube passes into a receiving flask containing a few drops of methyl red indicator and a known volume (the volume used depending upon the nitrogen content of the urine) of 0.1 N sulfuric acid. The end of the delivery tube must reach beneath the surface of the fluid.**[13] **Mix the contents of the distillation flask very thoroughly by**

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[10] There are numerous modifications of the original Kjeldahl method; the one described here, however, has given excellent satisfaction and is recommended for the determination of the nitrogen content of urine. With more resistant materials the addition of 10 g. of potassium sulfate in the acid digestion helps by raising the boiling point.

[11] This concentrated sodium hydroxide solution should be prepared in quantity and check tests should be made to determine the volume of the solution necessary to neutralize the volume (20 ml.) of concentrated sulfuric acid used. A few drops of phenolphthalein may be added prior to introducing the concentrated alkali to insure the addition of an excess.

[12] Powdered zinc may be substituted.

[13] This delivery tube should be of large caliber in order to avoid the sucking back of the fluid.

rotatory shaking and distil the mixture until its volume has diminished about one-half. Titrate the partly neutralized 0.1 N sulfuric acid solution by means of 0.1 N sodium hydroxide, and calculate the content of nitrogen of the urine examined.

CALCULATION. Subtract the number of ml. of 0.1 N sodium hydroxide used in the titration from the number of ml. of 0.1 N sulfuric acid taken. The remainder is equivalent to the number of ml. of 0.1 N sulfuric acid neutralized by the ammonia of the urine. One ml. of 0.1 N sulfuric acid is equivalent to 0.0014 g. of nitrogen. Therefore, if y represents the volume of urine used in the determination, and y' the number of ml. of 0.1 N sulfuric acid neutralized by the ammonia of the urine, we have the following proportion:

$y/100 = (y' \times 0.0014)/x$ in which x equals g. nitrogen per 100 ml. urine examined

Calculate the quantity of nitrogen in the 24-hour urine specimen.

Interpretation. An adult of medium size on a mixed diet will usually excrete 12 to 18 g. of nitrogen per day in the urine. On a low-protein diet the urinary nitrogen may drop to as low as 5 g. or so per day; on a high-protein diet, it may rise to 22 to 25 g. or even more. The total nitrogen determination includes the nitrogen from *all* the nitrogenous constituents of the urine; under ordinary circumstances the compound *urea* contributes about 80 per cent or more of the total nitrogen, the remainder being distributed between the various other nitrogenous constituents present (see below). In a normal adult, the total nitrogen of the urine and of the feces will often be almost exactly equal to the total nitrogen of the diet. Such a condition is called "nitrogen equilibrium." The feces usually contain very little nitrogen; it is a customary approximation in metabolic studies to assume that the fecal nitrogen will be 10 per cent of the urinary nitrogen.

CALCULATION OF PERCENTAGE NITROGEN DISTRIBUTION. In modern metabolism studies where the various forms of nitrogen are determined, in addition to the total nitrogen as yielded by the Kjeldahl method, it is customary to indicate what portion of the total nitrogen was present in the form of each of the individual nitrogenous constituents. These percentage values are secured by dividing the weight (grams) of nitrogen excreted for the day in the form of each individual nitrogenous constituent by the weight of the total nitrogen output for the same period. For example, if the total nitrogen excretion is 9.814 g. and the excretion of urea-nitrogen is 8.520 g. and the excretions of nitrogen in the forms of ammonia and creatinine are 0.271 g. and 0.639 g. respectively, the percentage distribution for these forms of nitrogen would be calculated as follows:

8.520 g. urea-nitrogen ÷ 9.814 g. total nitrogen = 84.3 per cent
0.271 g. ammonia-nitrogen ÷ 9.814 g. total nitrogen = 2.7 per cent
0.639 g. creatinine-nitrogen ÷ 9.814 g. total nitrogen = 6.5 per cent

NITROGEN PARTITION IN URINES CONTAINING ALBUMIN. If the urine to be tested contains albumin, this must be removed before an attempt at a nitrogen partition is made. This may be done by heating to boiling, acidifying with acetic acid to coagulate the protein, filtering, and making up the filtrate to the original volume of the urine. If very small amounts of albumin are present, removal is more difficult. In these cases the use of aluminum hydroxide cream is recommended since it removes none of the nitrogenous constituents of normal urine.

Procedure. One liter of urine (containing not over 1 per cent of albumin) is mixed with 1 liter of aluminum hydroxide cream[14] and filtered.

2. Folin-Farmer Micro-Kjeldahl Method:[15] *Principle.* This method belongs with the so-called microchemical methods inasmuch as it is adapted to the determination of amounts of nitrogen in the neighborhood of 1 mg. while in the ordinary Kjeldahl procedure 30 to 100 mg. of nitrogen is generally manipulated. One ml. of diluted urine is decomposed with sulfuric acid; as in the Kjeldahl method, the ammonia formed is set free by the addition of alkali, and carried over into an acid solution by means of a current of air. The ammonia solution is then treated with Nessler's reagent and the color produced compared with that of a standard solution of an ammonium salt treated in the same way. The reaction is

$$NH_4OH + 2(KI)_2HgI_2 + 3KOH \rightarrow NHg_2I + 7KI + 4H_2O$$

The solution of dimercuric ammonium iodide is colloidal. The conditions of formation must be carefully controlled to obtain uniformity and prevent precipitation. Chlorides decrease the color formation.

Procedure. Introduce 5 ml. of urine into a 50-ml. volumetric flask if the specific gravity of the urine is over 1.018, or into a 25-ml. flask if the specific gravity is less than 1.018.[16] Fill the flask to the mark with distilled water and invert it several times in order to guarantee thorough mixing. Transfer 1 ml. of the diluted urine to a large (20 to 25 mm. by 200 mm.) pyrex-glass test tube. To this add 1 ml. of concentrated sulfuric acid, 1 g. of potassium sulfate, 1 drop of 5 per cent copper sulfate solution, and a small, clean, quartz pebble or glass bead. (The pebble or bead is added to prevent bumping.) Boil the mixture over a microburner for about 6 minutes, i.e., about 2 minutes after the mixture has become clear and light green or almost colorless. Allow to cool until the digestion mixture begins to become viscous. This ordinarily takes about 3 minutes, but in any event the mixture must not be permitted to solidify. Add about 6 ml. of water (a few drops at a time, at first, then more rapidly) to prevent solidification. To this acid solution add an excess of sodium hydroxide solution (3 ml. of a saturated solution are sufficient), adding the alkali down the side of the tube so that it does not mix with the acid solution but forms a layer beneath it. Connect the tube with a suitable receiver in an aeration train (see Figs. 238 and 239) placing in the receiver[17] about 20 ml. of dilute (approximately 0.02 N) sulfuric acid. Start the air current (which mixes the alkali and acid in the digestion tube) and aspirate the

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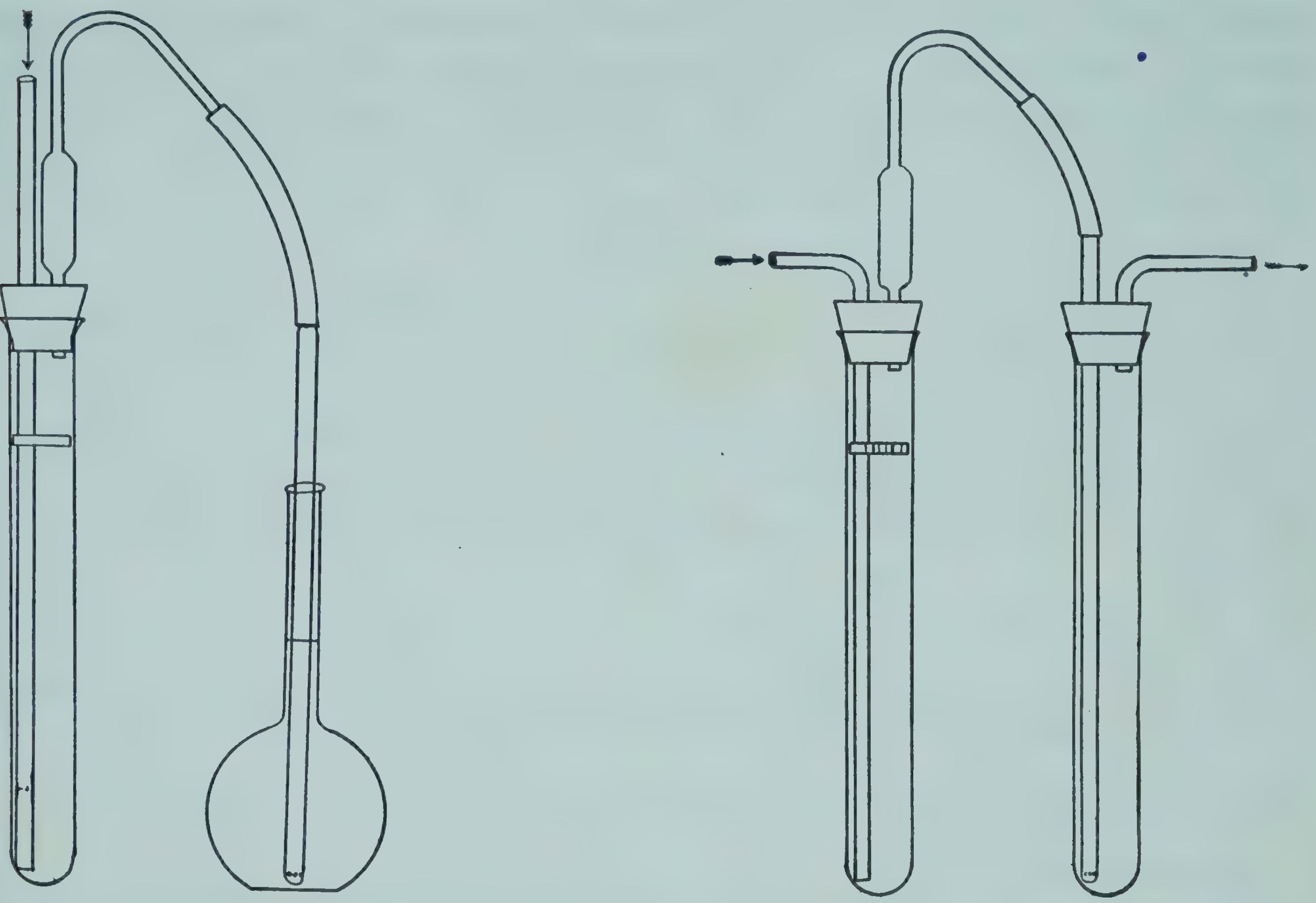
[14] See Appendix.

[15] Folin and Farmer: *J. Biol. Chem.*, **11**, 493 (1912).

[16] The purpose is to dilute the urine so that 1 ml. of the diluted fluid shall contain 0.75 to 1.5 mg. of nitrogen.

[17] For a single analysis, a volumetric flask is used as receiver, and compressed air is used for aspiration. For serial analyses, a receiving tube similar to the digestion tube and fitted with a two-hole rubber stopper carrying a long inlet and a short outlet tube is used. Either compressed air, or suction obtained from a water pump and applied at the end of the train, may be used. The entering air must be washed by passage through a wash bottle containing dilute (1:10) sulfuric acid, to remove any ammonia which may be present. As many pairs of tubes as desired may be connected in series. For method of cleaning rubber tubing used for connection, see p. 553. If suction is used, in disconnecting when aeration is complete, decrease the rate of air passage to a slow stream and start at the end farthest from the pump to avoid back pressure.

liberated ammonia into the receiving solution. The air current should be started slowly and should be only moderately rapid for the first 2 minutes, but at the end of this two-minute period the current should be run at its maximum speed for an interval of 8 minutes, or until all the ammonia has been driven over. The time required for complete aspiration of the ammonia will differ under different conditions and should be established by trial.[18]



FIGS. 238 (*left*) AND 239 (*right*). FORMS OF AERATION APPARATUS (FOLIN AND FARMER).

When all the ammonia has been aerated over, disconnect the receiver, dilute the contents to about 60 ml. with ammonia-free water in a 100 ml. volumetric flask (if such a flask has not itself been used as receiver), and dilute similarly 1 mg. of nitrogen in the form of ammonium sulfate[19] in a second volumetric flask. Nesslerize both solutions as nearly as possible at the same time with 10 ml. of Nessler solution[20] measured in a graduated cylinder and diluted, immediately before using, with about 20 ml. of ammonia-free water to avoid turbidity. Immediately fill the two flasks to the mark with ammonia-free water, mix well and allow to stand 5 minutes before

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[18] Run a trial analysis and have several receivers ready. After 8 minutes' aspiration, remove the first receiver and replace by a fresh one. Repeat at several later time intervals. Treat the contents of each receiver with Nessler solution, as described above for an analysis, and estimate from the color reaction the aeration time which gives complete transference of ammonia.

[19] Care should be taken to secure the pure salt. Ammonium salts may contain pyridine bases which titrate like ammonia but do not react with Nessler's reagent. Pure ammonium sulfate may be prepared by decomposing a high-grade ammonium salt with sodium hydroxide and passing the liberated ammonia into pure sulfuric acid. The salt is then precipitated by means of alcohol, brought into solution in water and then reprecipitated by alcohol. The final product should be dried in a desiccator over sulfuric acid. Pyridine-free ammonium salts are now obtainable on the market. A solution containing 0.4714 g. of ammonium sulfate in 1 liter of water, plus a few drops of concentrated sulfuric acid as preservative, contains 1 mg. of nitrogen in 10 ml. It is stable indefinitely.

[20] See Appendix.

reading in the colorimeter or photometer. For colorimetric comparison, match the standard against itself, and then the unknown against the standard, in the usual way (see p. 510). For photometric measurement, set the photometer to zero density (or 100 per cent transmittance) at 480 mμ (see below) with a blank prepared by diluting 10 ml. of Nessler reagent to 100 ml. with water. This blank will correct for any ammonia present in the diluting water, but not in the digestion reagents, etc. For more accurate results, run a blank and a standard through the entire analytical procedure (digestion, aeration, nesslerization), set the photometer to zero density with the blank, and obtain results in terms of the density of the analyzed standard. This should correct for ammonia in the reagents as well as systematic errors in the analysis.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. N in Standard} = \text{mg. N in volume of urine used}$$

At a 1:10 dilution of the urine, 1 ml. represents 0.1 ml. of urine; at a 1:5 dilution, 0.2 ml. of urine. Multiply the result of the above calculation by the dilution (10 or 5) to obtain the nitrogen content of the urine, in mg. per ml. or g. per liter (the two are identical). From this, the total nitrogen content of the 24-hour sample may be calculated.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. N in Standard} = \text{mg. N in volume of urine used}$$

Further calculation is similar to that described for colorimetric measurement.

Interpretation. See p. 875.

Spectrophotometric Characteristics of the Nessler Color with Ammonia. The color reaction between Nessler's reagent and ammonia is the basis for many other quantitative methods of biochemical importance, in addition to its use as described here for the determination of total nitrogen. The spectrophotometric characteristics of the Nessler color therefore deserve detailed consideration, since they determine the conditions for accurate photometric measurement, such as the choice of wavelength or filter, solution depth, and range of concentration over which Beer's law is applicable. The data presented here are based on nesslerization under the conditions described above, i.e., using 10 ml. of Nessler reagent in a final volume of 100 ml., in the absence of excess strong acid or alkali, or of protective substances such as gum ghatti. The values represent the conditions prevailing in the authors' laboratory at the time the analyses were made, and cannot be used for calibration purposes elsewhere, as discussed in Chapter 23, under "Colorimetry and Photometry," but may be used as a guide for such calibration.

The relation between the wavelength at which measurements are made, and light transmittance (optical density) for various amounts of ammonia nitrogen, measured at 1 cm. solution depth, is shown in Fig. 240A. There is no wavelength of peak or maximum light absorption in the visible portion of the spectrum for the amounts of nitrogen ordinarily encountered and under the prescribed conditions. The choice of wavelength therefore depends largely upon the sensitivity desired and the range of concentration over which Beer's law is applicable. This information is supplied by Fig. 240B, which represents the data of Fig. 240A, plotted in terms of the relation between transmittance or density and concentration at various wavelengths.

It is shown in Fig. 240B that in a 1-cm. cuvette (or 12.5-mm. test tube, since the two are approximately equivalent) amounts of nitrogen up to 1.5 mg. in 100 ml. of nesslerized solution will read within the accurate portion of the photometer scale (see p. 523) at any wavelength between 480 and 520 mμ. Such amounts may be determined by calculation based upon the density of a 1 mg. standard in accordance with Beer's law, as evidenced by the linearity of the "calibration curves" over this range. Above 520 mμ the sensitivity decreases and slight deviation from Beer's law is noted at higher concentrations of nitrogen. Below 480 mμ, agreement with Beer's law is excellent over the usable portion of the photometer scale, but the sensitivity is so great that only small amounts of nitrogen may be accurately measured. In a 2-cm. cuvette (a 22-mm. test tube is approximately equivalent to this) the same considerations apply, but at only half the concentration range specified for a 1-cm. cuvette. Under these conditions, the standard should contain 0.5 mg. of nitrogen, and the unknown not over 1 mg. of nitrogen per 100 ml. of nesslerized solution.

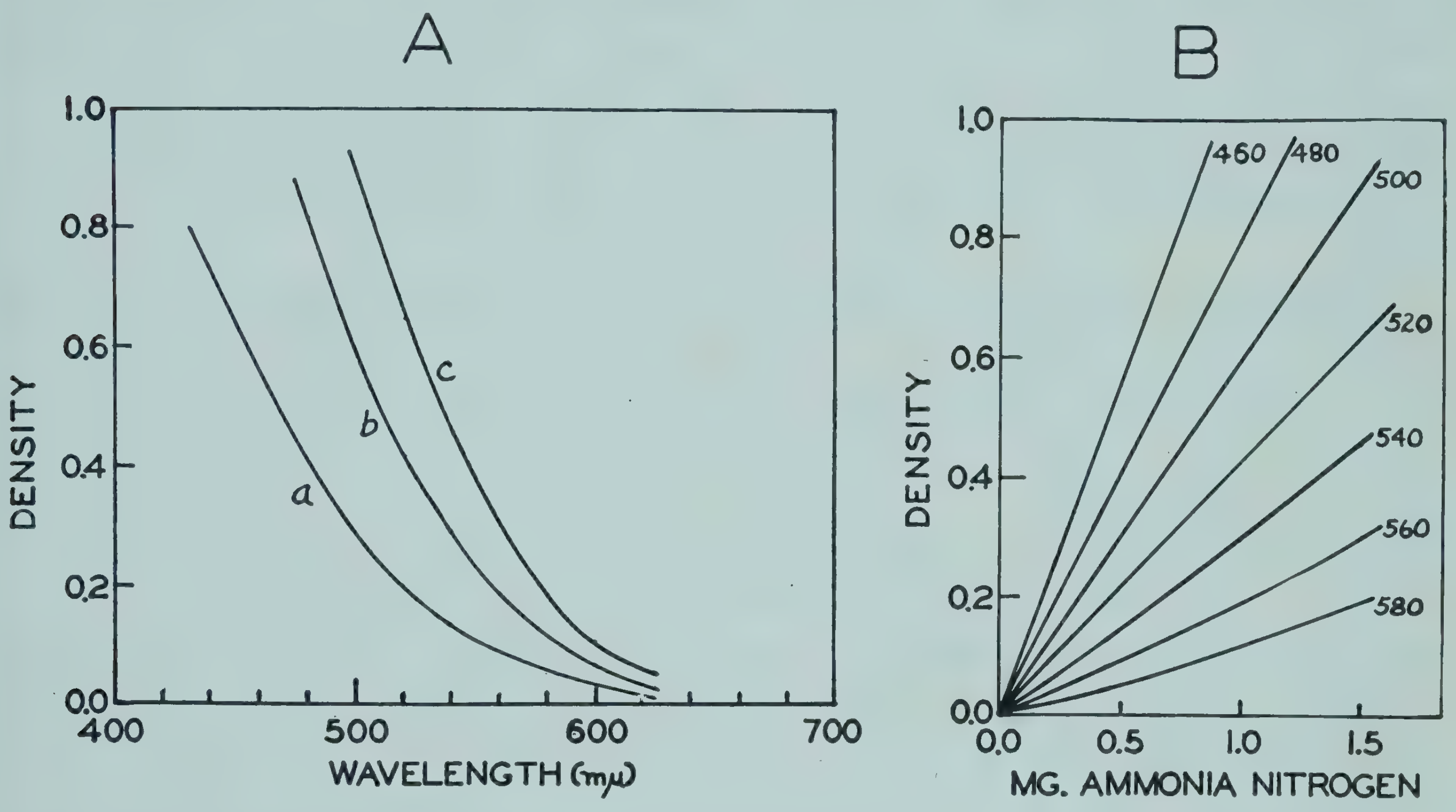

FIG. 240. SPECTROPHOTOMETRIC CHARACTERISTICS OF COLOR OBTAINED IN NESSLER REACTION WITH AMMONIA.

A, Relation between optical density and wave-length for solutions containing 0.5 mg. (*a*), 1.0 mg. (*b*), and 1.5 mg. (*c*), of ammonia nitrogen per 100 ml. final volume, at a solution depth of 1 cm. *B*, Relation between optical density and concentration at the wavelength indicated for each curve.

The proportionality between density and concentration with the wavelength or filter chosen should be tested for a particular photometer over the concentration range expected; significant deviation from Beer's law greater than that expected from the data of Fig. 240 usually indicates that either the photometer or filter is at fault. In this event, results must be based upon the actual calibration data obtained with the instrument; the use of such a "calibration curve" and the precautions which must be associated with its use, are discussed in Chapter 23.

Alternate Aeration Procedures with Titrimetric Estimation. Instead of nesslerizing the aerated ammonia for colorimetric estimation, one may aerate it into acid and make the determination by titration. Two such procedures are described. They have the advantage over the colorimetric method of permitting the accurate determination of larger amounts of nitrogen (up to 3 mg. or so). A disadvantage is that too vigorous aeration may carry over a small amount of the fixed alkali used to liberate the ammonia, and thus lead to titrimetric (but not colorimetric) error. To catch any spray, the bulb portion of the aeration outlet tube may be loosely packed with glass wool; in any event, the apparatus should be tested for this possibility of error by running a blank determination or analyzing a solution containing a known amount of nitrogen.

Aeration into Boric Acid. **Place 25 ml. of 2 per cent boric acid solution containing bromocresol green indicator, as described on p. 552 in connection with the determination of blood-urea nitrogen, in the receiving flask or test tube. Liberate the ammonia from the digest by alkali as described for the Folin-Farmer method, and aerate the liberated ammonia into the boric acid solution. When aeration is complete, remove the receiver and titrate the contents with 0.0143 N sulfuric acid (see p. 552) until the more or less blue color is exactly restored to its original yellow-green shade, as evidenced by matching against a control 25-ml. portion of boric acid plus indicator which is diluted with water to approximately the same final volume as the titrated sample. The end point should be sensitive to about 0.02 ml. of the standard acid.**

CALCULATION. Each ml. of 0.0143 N acid is equivalent to 0.2 mg. of nitrogen. Multiply the number of milliliters of 0.0143 N acid used by 0.2 to give milligrams of nitrogen in the volume of urine used.

Aeration into Standard Acid. **Aerate the liberated ammonia into a receiver containing 25 ml. of 0.01 N sulfuric acid. When aeration is complete, remove the receiver, add a drop of methyl red indicator solution, and titrate with 0.01 N sodium hydroxide to an orange or beginning yellow shade.**

CALCULATION. Subtract the number of ml. of 0.01 N alkali used from 25.0, to obtain the amount of 0.01 N acid neutralized by the ammonia. Multiply this value by 0.14 to obtain the nitrogen content in mg. of the volume of undiluted urine used in the analysis, since each ml. of 0.01 N acid repesents 0.14 mg. of nitrogen.

3. Determination of Nitrogen by Micro-Kjeldahl and Distillation. Instead of aerating over the ammonia of a micro-Kjeldahl determination, it may be distilled over and collected in acid for subsequent determination by colorimetric or titrimetric methods. In general, more accurate and consistent results are obtained by distillation than by aeration. Bock and Benedict have described a simple distillation procedure using an ordinary small Liebig condenser.[21] For research work, steam distillation has largely replaced simple distillation, and is the method of choice for all accurate micro-Kjeldahl analyses. Modern steam-distillation apparatus (see Fig. 241) is compact, easy to use, almost automatic in operation, and requires but a few minutes for each sample.

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[21] Bock and Benedict: *J. Biol. Chem.*, **20**, 47 (1915).

Procedure. **Digest the sample as described for the Folin-Farmer method, p. 876; or digest it with 1 ml. of concentrated sulfuric acid plus a few small grains of metallic selenium or a "Hengar granule" (a quartz chip coated with selenium).[22] Digestion must be continued until all of the nitrogen has been converted to ammonia; the time required for complete conversion varies with different types of material and should be established by trial.**

Transfer the digested sample with rinsings to the chamber of the steam-distillation apparatus, which has previously been cleared of any contaminating ammonia by a blank distillation. Place the receiving fluid (this varies with the procedure, see below) in a small flask arranged so that the tip of the condenser outlet dips below the surface of the receiving fluid. Add sufficient concentrated sodium hydroxide to the digest in the chamber to more than neutralize the amount of acid present (determine by trial). Start the generation of steam in the boiler, and steam-distil the sample until 8 to 10 ml. or more of distillate have been collected in the receiving flask. Remove the receiver, rinsing down the sides of the condenser outlet tube with a little water in the process, and determine the ammonia in the distillate by any of the following procedures.[23]

(a) COLORIMETRIC OR PHOTOMETRIC ESTIMATION. **Use 1 to 2 ml. of dilute (0.1 N) acid in the receiving flask and collect the distillate as described. Transfer the distillate to a container graduated at 25 ml., with rinsings up to about 20-ml. volume. Add 2.5 ml. of Nessler solution, dilute to 25 ml. with water, and mix. Compare in a colorimeter or photometer with a standard containing 0.2 mg. of nitrogen, prepared in a second 25-ml. container in the same way as for the distillate. In photometric measurement, if the standard is digested and distilled as for the sample, and the photometer is set to zero density with a digested and distilled blank, correction is automatically obtained for any systematic error or for ammonia in the reagents.**

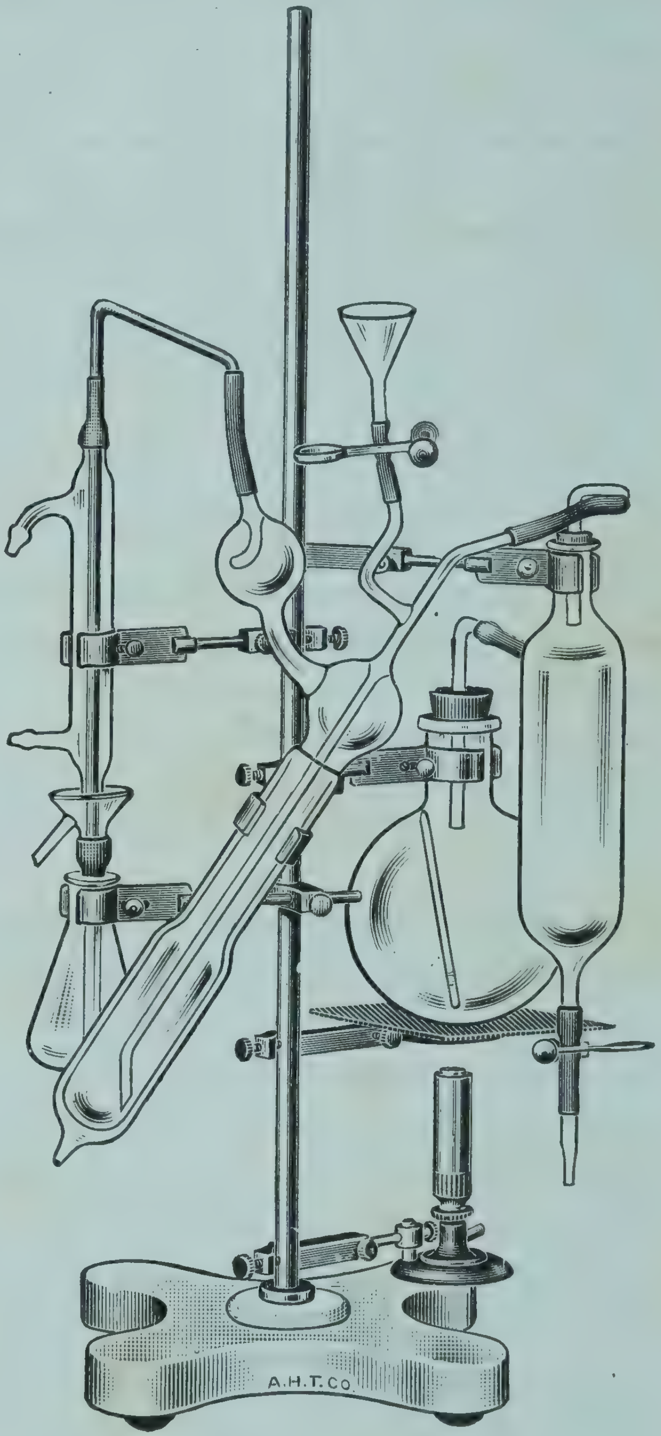

FIG. 241. STEAM DISTILLATION APPARATUS.

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[22] Obtainable from the Hengar Co., Philadelphia, or from dealers in laboratory supplies.

[23] On disconnection or diversion of the steam flow from the chamber of the apparatus, the residue of the sample will usually be removed automatically by siphoning in a few moments as the chamber cools down. The apparatus is then ready for the next sample, without further rinsing.

CALCULATION. Similar to that given for the Folin-Farmer procedure, p. 876. The result gives the mg. of nitrogen present in the portion of sample analyzed. The conditions for photometric measurement have been presented on p. 878. For accurate measurement, not more than 0.5 mg. of nitrogen may be present if a 1-cm. cuvette is used, or half this amount with a 2-cm. cuvette. Much smaller amounts than this (down to 0.01 mg. or less) may be accurately estimated by using 1 ml. of receiving acid in a test tube graduated at 10 ml., collecting the distillate up to the 10-ml. mark, adding 1 ml. of Nessler solution, and comparing photometrically with a suitable standard.

(b) TITRIMETRIC ESTIMATION. **Place 10 ml. of 2 per cent boric acid, containing bromocresol green indicator as described on p. 552 for the determination of blood urea nitrogen, in the receiving flask, collect 10 to 15 ml. of distillate as described, and titrate the contents of the receiving flask with 0.0143 N (or 0.02 N) sulfuric acid until the solution has been brought back to its original yellow-green color. Match against a 10-ml. control portion of boric acid-indicator diluted with water to approximately the final volume of the titrated sample.**

CALCULATION. Multiply the number of ml. of 0.0143 N (or 0.02 N) acid used by 0.2 (or 0.28) to obtain the mg. of nitrogen in the portion of sample used. Titration of a digested and distilled standard and blank will serve as a basis for correction for any ammonia present in the reagents. Amounts of nitrogen from 0.05 to 3 mg. or more may be accurately determined by this procedure. Alternatively, the distillate may be collected in 25 ml. of 0.01 N acid and the excess acid back-titrated with 0.01 N alkali in the presence of methyl red indicator, as described under the Folin-Farmer procedure. The boric acid method is preferred because it is a direct titration and requires only one standard solution, which is quite stable.

4. Other Methods. Many other modifications of the macro-Kjeldahl and micro-Kjeldahl determination of total nitrogen have been proposed; those described here are believed to be as satisfactory as any. For micro-Kjeldahl analyses, direct nesslerization of the diluted digest has been proposed by Folin and Denis and by Koch and McMeekin; these procedures are similar in details to the direct nesslerization methods for the determination of blood nonprotein nitrogen, as described in Chapter 23. In general, direct nesslerization is less satisfactory for urinary nitrogen than for blood nonprotein nitrogen, because of the difficulty of avoiding turbidity. Other methods of determining nitrogen include those based upon gasometric estimation; their use with urine is similar to that for blood nonprotein nitrogen, and references may be found in this connection in Chapter 23.

UREA

1. UREASE METHODS

These methods depend upon the principle that the enzyme urease is able, at ordinary temperatures, to transform urea, quickly and completely, into ammonium carbonate. Takeuchi in 1909 discovered the presence of this enzyme in the soja or soy bean. The application of this enzyme to the determination of urea in urine, blood, etc., was first proposed by Marshall, whose methods have been modified by Van Slyke and Cullen. These latter investigators prepared a permanent preparation of the enzyme, in a water-soluble form, the use of which makes more convenient the rapid and accurate determination of urea in urine, blood, and other biological fluids.

The urease method is probably the most satisfactory of all methods for the determination of urea. Other nitrogenous constituents such as allantoin are not decomposed by urease. The method involves no carefully regulated heating procedures, and is applicable to diabetic urines. Since, however, the basis for the urea determination is essentially an analysis for the ammonia produced by the action of the enzyme, every analysis will include the preformed ammonia which is also present in the urine, so that uncorrected results represent *urea plus ammonia.* To obtain the urea content, it is necessary to know the amount of preformed ammonia present, as established by separate analysis, and to subtract this from the results of the urea determination. Urea (and ammonia) determinations should be carried out on fresh or recently collected and preserved samples of urine, since on long standing, even in the presence of preservatives, significant amounts of urea may undergo hydrolysis to form ammonia, thus leading to misinterpretation of the significance of both the urea and the ammonia values. Kinetic studies have shown that a high concentration of urea or ammonium ion tends to inhibit the action of urease.[24] The conversion of urea to ammonia may take place quite rapidly in urine contaminated with bacteria.

a. Method of Van Slyke and Cullen: Principle. The urine sample is treated with urease, and the ammonia formed is aerated into 0.02 N acid, which is then back-titrated with 0.02 N alkali. A modification based on aeration into boric acid and direct titration with standard acid is also described; this is preferred over the original method because just as accurate results are obtained and only one standard solution is required in a direct titration.

PREPARATION OF SOLID UREASE.[25] Digest 1 part of jack-bean meal[26] with 5 parts of water at room temperature, with occasional stirring, for an hour, and clear the solution by filtration through paper pulp or centrifuging. Pour this extract slowly, with stirring, into at least 10 volumes of acetone. The acetone dehydrates the enzyme preparation. Filter, dry in vacuum, and powder. The activity of the preparation is retained indefinitely. Thus prepared it is not perfectly soluble in water, but this fact interferes in no way with its use.

STANDARDIZATION OF THE ENZYME PREPARATION. Make up accurately a 3 per cent solution of pure urea. Treat this solution exactly as the urine is treated in the following method, using 0.5 ml. of the solution. The ammonia formed should neutralize 25 ml. of 0.02 N acid, or an equivalent amount of acid of other strength. If it does so the preparation is of sufficient strength to use as indicated. If not, more of the preparation must be used for a determination.

Procedure. **Dilute 5 ml. of urine to 50 ml. with ammonia-free water. Measure 5 ml. of the diluted urine into a large test tube suitable for aeration** (see **Fig. 238, p. 877, and also Fig. 147, p. 552), add 1 drop of caprylic alcohol** (to

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[24] Laidler and Hoare: *J. Am. Chem. Soc.*, **71**, 2699 (1949).

[25] Van Slyke and Cullen: *J. Biol. Chem.*, **19**, 211 (1914). Dry urease prepared in this way is available commercially in powder and tablet form.

[26] Watermelon seeds have also been shown to be an excellent source of urease (Damodaran and Sivaramakrishnan: *Biochem. J.*, **31**, 1041 (1937)).

prevent frothing), and 1 ml. of enzyme solution.[27] Close the tube with a two-hole rubber stopper fitted with a long inlet tube and a short outlet tube, as illustrated in the figures cited, and let the tube stand 15 minutes for the enzyme to act. Measure into a second similar tube 25 ml. of 0.02 N HCl or H_2SO_4. Add 1 drop of caprylic alcohol and 1 drop of methyl red indicator solution. Connect the two tubes for aeration with washed air by either pressure or suction, as described on p. 876. At the end of 15 minutes aspirate for about one-half minute to transfer any ammonia present in the free condition to the receiving solution. After this aspiration, open the tube containing the sample and introduce 5 ml. of saturated potassium carbonate. Close the tube at once and aspirate until all the ammonia has been carried over into the acid in the receiver. The time needed for the aspiration varies for different pumps from 5 to 30 minutes, and should be determined by trial for the particular apparatus used. At the end of the time needed for the aeration, the pump is disconnected (care being taken to avoid back suction) and the excess acid in the receiver is titrated by means of 0.02 N NaOH.

CALCULATION. Subtract the number of ml. of 0.02 N alkali required for the titration from 25.0 to obtain the volume of 0.02 N acid equivalent to the ammonia present. Since 1 ml. of 0.02 N acid is equivalent to 0.28 mg. of nitrogen, and 0.5 ml. of urine is used in an analysis (5 ml. of a 1:10 dilution), multiply the volume of 0.02 N acid found equal to the ammonia by 0.56 (= 2 × 0.28) to obtain the *urea plus ammonia* nitrogen content, in g. per liter (mg. per ml.). Subtract from this value the ammonia-nitrogen content, in g. per liter, as established by a separate analysis (see p. 888), to obtain the urea-nitrogen content of the urine, in g. per liter. From this, and the total volume of the sample, the urea-nitrogen content of the 24-hour sample is readily obtained.

Aeration into Boric Acid. **Proceed exactly as described above, but aerate the ammonia into 25 ml. of the 2 per cent boric acid-indicator solution described on p. 552 in connection with the determination of blood-urea nitrogen (see also the modified Folin-Farmer method, p. 880). When aeration is complete, titrate the boric acid solution with 0.0143 N (or 0.02 N) sulfuric acid until the more or less blue color has been restored to the original yellow-green color, as established by comparison with a control 25-ml. portion of boric acid-indicator solution which is diluted with water to approximately the same final volume as the titrated sample. The end point should be sharp to about 0.02 ml. of the standard acid.**

CALCULATION. Each ml. of 0.0143 N acid is equivalent to 0.2 mg. nitrogen; if 0.02 N acid is used, 1 ml. equals 0.28 mg. nitrogen. Multiply the volume in ml. of acid required for the titration by 0.2 (or 0.28), and then further by 2, to obtain the urea plus ammonia nitrogen content of the urine, in g. per liter. Subtract the ammonia-nitrogen content of the urine, determined separately, to obtain the urea-nitrogen content.

Interpretation. The mean average daily excretion of urea by normal adults is usually placed at about 25 to 35 g. (10 to 15 g. of urea nitrogen) but is very closely dependent upon the protein ingestion and metabolism and hence may vary widely. In disorders associated with increased tissue catabolism, as in fevers, the excretion of urea is increased. It may be decreased in pronounced kidney and liver disorders due to decreased formation and decreased power of elimination, but these findings are not constant. The determination of urinary urea, in conjunction with the determination of blood urea, is, however, of major clinical value as an index of kidney function (see urea clearance test, p. 965).

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[27] The enzyme solution is prepared by dissolving 2 g. of the enzyme preparation, 0.6 g. of dipotassium phosphate, and 0.4 g. of monopotassium phosphate in 10 ml. of water. Solution is aided by stirring with a glass rod. The slightly opalescent solution should be covered with toluene and may be kept for two weeks without losing activity.

The percentage of the total nitrogen of the urine occurring as urea varies on the average from 80 to 90. On a high-protein diet it is nearer 90 per cent; on a very low-nitrogen but high-calorie diet it may not be over 60 per cent. In marked acidosis it may be considerably decreased relative to the total nitrogen (see "Ammonia," p. 888).

b. Direct Nesslerization Method (***Folin and Youngburg***[28] ***Modified***)***: Principle.*** The diluted urine is treated with an alcoholic urease solution to convert urea into ammonia, and the ammonia present is then determined by direct nesslerization. In the original procedure, preformed ammonia is removed by treatment with Permutit (a synthetic "exchange silicate," see p. 890) and the determination carried out on the ammonia-free solution. It has been found, however, that Permutit may at times remove a significant amount of the urea as well, and its use has been dispensed with in the modification described here. The total urea plus ammonia nitrogen is determined by direct nesslerization after urease treatment; the preformed ammonia content of the urine, as determined by a separate analysis (p. 888) is subtracted from the result to give the urea-nitrogen content of the urine.

Direct nesslerization is perhaps not quite so accurate as the aeration procedures because of the possibility of turbidity produced by interfering substances in the color reaction, but the procedure has the advantage of simplicity and the results are quite suitable for most purposes.

Procedure. **Dilute 5 ml. of urine to 100 ml. in a volumetric flask and mix well. Transfer 1 ml. of the diluted urine to a test tube, add 1 ml. of the alcoholic urease solution,[29] and 1 drop of buffer solution.[30] Digest in a beaker of warm water (40° to 55° C.) for 5 minutes or at room temperature for 15 minutes, at the end of which time transfer the contents of the test tube, with rinsings, to a 100-ml. volumetric flask, diluting to a volume of about 80 ml. Prepare a standard in a second flask by adding sufficient standard nitrogen solution to contain 0.5 mg. of nitrogen,[31] 1 ml. of urease solution, and water to a volume of about 80 ml. To each flask add 1 ml. of gum ghatti solution,[32] set the flask contents to swirling and add from a graduated cylinder 10 ml. of Nessler reagent.[32] Dilute immediately to 100 ml. mix, and allow to stand 5 minutes. Read in a colorimeter or photometer in the usual way. For photometric measurement, set the photometer to zero density with a blank prepared similarly to the standard except that the standard nitrogen solution is omitted.**

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{mg. N in}}{\text{Standard}} \times \text{Dilution} = \begin{array}{c}\text{mg. urea- plus ammonia-nitrogen}\\ \text{in 1 ml. of undiluted urine}\end{array}$$

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[28] Folin and Youngburg: *J. Biol. Chem.*, **38**, 111 (1919); and Youngburg: *J. Biol. Chem.*, **45**, 391 (1921).

[29] To prepare the alcoholic urease solution place 3 g. of Permutit in a flask, wash once with 2 per cent acetic acid, then twice with water; add 5 g. of fine jack-bean meal and 100 ml. of 30 per cent alcohol. Shake gently but continuously for 10 to 15 minutes and filter. The filtrate contains practically all of the urease and extremely little of other materials.

[30] Dissolve 14.2 g. of Na_2HPO_4 and 12.0 g. of NaH_2PO_4, or equivalent amounts of the crystalline salts, in water and make up to 100 ml.

[31] See footnote 19, p. 877.

[32] See Appendix.

Subtract from this value the ammonia content of the urine, as determined separately, to obtain the urea-nitrogen content of the urine. Under the conditions described, the dilution is 20, and urines containing 5 to 20 g. of urea nitrogen per liter may be accurately analyzed. For values outside this range, repeat the analysis at a more satisfactory dilution.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \frac{\text{mg. N in}}{\text{Standard}} \times \text{Dilution} = \begin{array}{c}\text{mg. urea- plus ammonia-nitrogen}\\ \text{in 1 ml. undiluted urine}\end{array}$$

Correct for the preformed ammonia content as described above. The conditions for photometric measurement of the Nessler color have already been described (p. 878). At 480 mμ, and in a 1-cm. cuvette, the standard has a density of approximately 0.400, permitting measurement with urines containing up to about 25 g. of urea nitrogen per liter under these conditions. For higher values, or for photometric measurement at greater depth of solution, use a greater dilution of the urine.

Interpretation. See p. 884.

*c. **Microdiffusion Method of Conway:***[33] ***Principle.*** The sample of diluted urine is incubated with urease in the outer compartment of a "Conway diffusion cell" (Fig. 242).[34] The ammonia formed is then liberated by addition of saturated potassium carbonate solution, and the cell is covered. The ammonia diffuses over into and is absorbed by acid in the inner compartment of the cell, where it may there be determined by titrimetric or colorimetric means. As with all urease methods for urine, separate determination of the ammonia N present is necessary in order to obtain the urea N content.

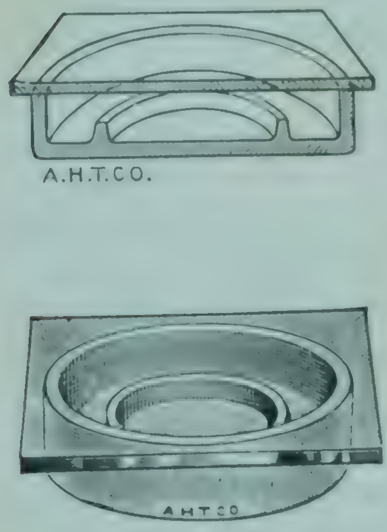

FIG. 242. CONWAY CELL.

The same principle may be employed for the determination of urea N in blood, of total N in micro-Kjeldahl digests, and in general wherever a volatile absorbable substance is to be determined (see p. 668). Because of the simplicity of the procedure and its application to microanalysis, increasing use is being made of this principle in routine and research laboratories.

Procedure: Determination of Urea N Plus Ammonia N. **Dilute 1 ml. of urine to 10 ml. with water and mix. With an accurate pipet transfer 0.2 ml. of this diluted urine to the outer compartment of a Conway diffusion cell (Fig. 242). Place 1 ml. of 2 per cent boric acid solution containing added bromocresol green indicator**[35] **in the inner compartment. Smear a glass cover plate with a**

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[33] Conway and Byrne: *Biochem. J.*, **27**, 419 (1933); Conway: *ibid.*, **27**, 430 (1933). The procedure as described here is a slight modification of the original Conway procedure, and includes some suggestions of Steinitz (*J. Lab. Clin. Med.*, **25**, 288 (1939–1940)). For application of the microdiffusion principle to the determination of small amounts of nitrogen in tissue preparations, see Borsook: *J. Biol. Chem.*, **110**, 481 (1935). For application to the determination of acetone in blood and urine, see Werch: *J. Lab. Clin. Med.*, **25**, 414 (1939–1940); **26**, 878 (1940–1941).

[34] The unit shown here is made of Coors porcelain and may be obtained from the Arthur H. Thomas Co., Philadelphia.

[35] See p. 552 for preparation of this solution.

thin film of fixative[36] and place on the cell, greased side down. Slide the cover aside far enough to permit the introduction of a pipet tip, and add to the outer chamber 0.5 ml. of urease-phosphate solution.[37] Replace the cover, and mix the fluids in the outer chamber by slight tilting and rotation of the cell. Set aside at room temperature for 15 minutes. At the end of this time, tilt the cell slightly to displace the fluid in the outer chamber to one side, slide the cover aside slightly to permit the introduction of a pipet at a point opposite to the displaced fluid, and add 1 ml. of saturated potassium carbonate solution to the outer chamber. At once replace the cover, mix the fluids in the outer chamber by tilting and rotation as above, and set aside for 90 minutes at room temperature (or place in an incubator at 38° C. for 1 hour). Be sure the cover is firmly in place during this period. At the end of the period, remove the cover and titrate the contents of the inner chamber with 0.0143 N sulfuric acid,[38] using a microburet with a fine tip which dips below the surface of the solution being titrated. Titrate until the color of the fluid in the inner chamber exactly matches that in a control cell. The control consists of a second cell treated exactly as described for an analysis except that 0.5 ml. of water replaces the urine sample.

Ammonia N Determination. **Proceed as above, but use 0.2 ml. of undiluted urine and omit the treatment with urease, adding the 1 ml. of saturated potassium carbonate solution directly to the urine in the outer compartment, allowing to stand covered for diffusion and absorption of ammonia to take place, titrating, etc., as described.**

CALCULATION. Since 1 ml. of 0.0143 N sulfuric acid is equivalent to 0.2 mg. of nitrogen, the calculation is as follows:

a. Urea N + Ammonia N:

ml. 0.0143 N acid required × 0.2 × 50 = mg. urea N + NH_3 N per ml. undiluted urine

b. Ammonia N:

ml. 0.0143 N acid required × 0.2 × 5 = mg. NH_3 N per ml. undiluted urine

c. Urea N: subtract (b) from (a) to obtain the urea N content of the urine, in mg. per ml. (or g. per liter).

During the titration, the fluid in the inner compartment should be stirred continuously; a convenient arrangement consists of a fine glass tip delivering compressed air in a slow stream of bubbles below the surface of the solution. A magnetic stirrer acting on a "flea" (small piece of iron wire enclosed in a small sealed glass tube) placed in the inner compartment is also an excellent method of stirring during the titration. If desired, the determination may be made colorimetrically or photometrically by placing 1 ml. of 0.1 N sulfuric acid (without indicator) in the inner compartment, instead of the boric acid solution. After absorption of ammonia is complete, the contents of the inner chamber are transferred by means of a rubber-bulb pipet, with rinsings, to a test tube graduated at 25 ml. and nesslerized at this volume in the presence of 2.5 ml. of Nessler reagent. Colorimetric or photometric estimation may then be made in terms of a standard containing 0.2 mg. of nitrogen, nesslerized similarly.

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[36] Ordinary petrolatum is satisfactory at room temperatures. If the cells are to be incubated at 38° C., a petrolatum-paraffin wax mixture is recommended. Melt 3 parts petrolatum with 1 part paraffin wax (M.P. 55°) and allow to cool.

[37] Dissolve 400 mg. of urease powder (prepared as described on p. 883; commercial preparations may also be used) in 10 ml. of water and add 0.4 ml. of phosphate buffer (6.9 g. of $NaH_2PO_4 \cdot H_2O$ and 17.9 g. of $Na_2HPO_4 \cdot 12H_2O$ in 100 ml. of water). Prepare the urease solution fresh daily, and discard any unused portion. The activity of the powder may be tested by a control analysis on pure urea solution. Dilute a 2.14 per cent solution of urea 1:10 with water and analyze 0.2 ml. of the diluted solution; 0.2 mg. of nitrogen should be found.

[38] Dilute 14.3 ml. of N sulfuric acid to 1 liter in a volumetric flask and mix. This solution is quite stable.

Interpretation. See previous methods.

2. OTHER METHODS

Many other methods for the determination of urinary urea content have been described, some of which combine certain features of the various procedures described here. Rose and Coleman suggested colorimetric determination of the aerated ammonia (as in the Folin-Farmer micro-Kjeldahl method, p. 876), after treatment of the urine with urease. Marshall described a simple clinical method based upon direct titration of the ammonia liberated by urease treatment.[39] Gasometric measurement in the Van Slyke-Neill apparatus (p. 709) of the carbon dioxide liberated by the action of urease on urea[40] is simple, rapid, and accurate; the only disadvantage is when many analyses are to be done, since each analysis must be carried to completion before the next is begun. Gasometric determination based on the liberation of nitrogen by treatment with hypobromite has been described by Stehle.[41] Methods based upon isolation of urea as the insoluble xanthydrol compound have been described by Barrett and Jones[42] and by Allen and Luck.[43] Direct colorimetric methods which do not involve the action of urease have been proposed by Ormsby,[44] Barker,[45] and Archibald.[46]

AMMONIA

Introduction. Many of the methods which have been proposed for the determination of urinary ammonia are based upon principles similar to those already described in connection with the micro-Kjeldahl determination, and the determination of urinary urea, since these consist essentially in the determination of ammonia produced as a result of the analytical treatment. For the accurate determination of urinary ammonia, it is necessary to separate the ammonia from interfering material, and to avoid the use of excessive heat or strong alkali in the treatment of the urine, since these may lead to the production of ammonia from other nitrogenous constituents of the urine. The successful separation of ammonia from urine by aeration was first described by Folin;[47] his method was modified and improved by Van Slyke and Cullen, and their method (with modification) is described here, since it requires less urine than Folin's method, gives just as accurate results, and utilizes the same apparatus, reagents, and technique as have already been described for the determination of urea in blood (p. 551) and in urine (p. 883).

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[39] Marshall: *J. Biol. Chem.*, **14**, 283 (1913).

[40] Van Slyke: *J. Biol. Chem.*, **73**, 695 (1927).

[41] Stehle: *J. Biol. Chem.*, **47**, 13 (1921), and **51**, 89 (1922). See also Van Slyke: *J. Biol. Chem.*, **83**, 449 (1929).

[42] Barrett and Jones: *Biochem. J.*, **26**, 1246 (1932).

[43] Allen and Luck: *J. Biol. Chem.*, **82**, 693 (1929).

[44] Ormsby: *J. Biol. Chem.*, **146**, 595 (1942).

[45] Barker: *J. Biol. Chem.*, **152**, 453 (1944).

[46] Archibald: *J. Biol. Chem.*, **157**, 507 (1945).

[47] Folin's method is described in the Eleventh Edition of this book.

1. Aeration Method (*Van Slyke and Cullen, Modified*): ***Principle.*** The urine is treated with an equal volume of saturated potassium carbonate solution, and the liberated ammonia is transferred by aeration into an acid receiving solution, where it is then determined by titration. In the original procedure, the ammonia is aerated into 0.02 N acid which is then back-titrated with 0.02 N alkali. In the modification described here, boric acid solution is used to receive the ammonia, which is then titrated directly with standard acid. Results are the same by either procedure; the boric acid method has the advantage of being a direct titration and requires only one standard solution which is quite stable.

Procedure. **Measure 5 ml. of undiluted urine into one of the two large test tubes used in an aeration train (see Fig. 238, p. 877, and Fig. 147, p. 552) and connect this tube for aeration, as shown in the illustrations, with a second tube containing 25 ml. of the 2 per cent boric acid containing added bromocresol green indicator, described on p. 552 in connection with the determination of blood-urea nitrogen. Add a drop of caprylic alcohol to each tube to minimize foaming. When ready, remove the stopper of the tube containing the urine and add 5 ml. of saturated potassium carbonate solution. Replace the stopper tightly and start the air current (pressure or suction; the incoming air must be washed by preliminary passage through a wash-bottle containing dilute (1:10) sulfuric acid, to remove any ammonia present). The air current should be run slowly for the first two minutes, and then increased to a rate as fast as the apparatus will stand. Aeration is continued until all the ammonia has been driven over; this may take from 5 to 30 minutes depending upon the apparatus, and the time required should be established by trial.**

When aeration is complete, remove the tube containing the boric acid, rinsing down the inlet tube in the process, and titrate the contents with 0.0143 N (or 0.02 N) sulfuric acid, until the more or less blue color is replaced by the original yellow-green color, as determined by matching against a control 25-ml. portion of the boric acid indicator solution which has been diluted with water to approximately the final volume of the titrated sample. The end point should be sharp to about 0.02 ml. of standard acid.

CALCULATION. Each ml. of 0.0143 N acid is equivalent to 0.2 mg. of ammonia nitrogen; if 0.02 N acid is used, 1 ml. equals 0.28 mg. of ammonia nitrogen. Multiply the volume in ml. of acid required for the titration by 0.2 (or 0.28), to obtain the ammonia-nitrogen content of 5 ml. of urine; divide the result by 5 to obtain the ammonia-nitrogen content of the urine in mg. per ml. (or g. per liter). Results on any other basis (e.g., g. per 100 ml. or per 24-hour sample) may then be readily obtained.

If the original Van Slyke-Cullen procedure based on back-titration of 0.02 N acid with 0.02 N alkali is used, the conditions and the calculations are similar to those described on p. 883 in connection with the determination of urinary urea, except that results are divided by 10 because 10 times as much urine is used for the ammonia determination as for the urea determination.

The ammonia aerated into dilute (0.02 N) sulfuric acid may be nesslerized and determined colorimetrically or photometrically as described for the Folin-Farmer micro-Kjeldahl procedure, p. 876; this procedure was suggested by Folin and MacCallum. The amount of urine taken should not contain over 0.5 to 1.0 mg. of ammonia nitrogen, i.e., 1 to 2 ml. of normal urine, or less in acidosis, diluted to about 5 ml. with water. Comparison is made against a suitable standard, nesslerized in the same way, and the calculations are similar to those for the Folin-Farmer procedure.

Interpretation. The average daily output of ammonia nitrogen in the urine of an adult on a mixed diet is about 0.7 g., corresponding to about 50 milliequivalents, or 500 ml. of 0.1 N base, per day, and representing about 2.5 to 4.5 per cent of the total nitrogen. The amount excreted may vary widely from this average value, however, since (along with the titratable acidity of the urine, see p. 869) the ammonia content of the urine appears to parallel the state of acid-base balance within the body. It is increased in amount by the ingestion of acids or acid-forming foods and in the acidosis of starvation or diabetes (but not of nephritis), and decreased by the ingestion of alkalies or base-forming foods and in alkalosis. The ammonia of the urine is liberated enzymatically in the kidneys principally from glutamine but also from amino acids and other possible precursors. Urinary ammonia appears to function as a "synthetic" base, capable of replacing "fixed base" (sodium, potassium) in the excretion of acids as their neutralized salts, and thus conserving such fixed base to the organism.

2. Formol Titration Method** (**Malfatti**): **Principle. This method is based on the reaction that takes place when formalin solution is added to a solution containing ammonium salts (see "Amino Acid Nitrogen" methods). An acid reaction is produced in the mixture, which is then titrated with standard alkali using phenolphthalein as an indicator. Amino acids give the same reaction so that the result of the titration represents ammonia + amino acid nitrogen. This method may be used for the rapid clinical estimation of these forms of nitrogen as a substitute for an ammonia determination, but the results do not represent ammonia as is sometimes stated.

Procedure. **To 25 ml. of urine in a 200-ml. Erlenmeyer flask add 5 g. of finely pulverized potassium oxalate, a few drops of phenolphthalein, and titrate to a faint but permanent pink color with 0.1 N NaOH. (The urine mixture just after neutralization in the urinary titratable acidity determination may be used.) (See p. 870.) Then add 10 ml. of neutral formalin solution (see "Amino Acid Nitrogen"), mix well, and titrate with 0.1 N sodium hydroxide to a permanent pink color.**

CALCULATION. One ml. of 0.1 N sodium hydroxide is equivalent to 1.7 mg. of ammonia. Multiply the number of ml. of 0.1 N alkali required after the addition of the formalin by 1.7 and by 4 to get the number of mg. of ammonia + amino acid nitrogen (expressed as ammonia) in 100 ml. of the urine examined.

3. Permutit Method:**[48] **Principle. The urine is shaken with particles of an "exchange silicate," which remove the ammonia from solution. The ammonia is set free from the silicate by treating with alkali solution. This is then nesslerized and compared with a standard ammonia solution nesslerized in the same way.

Procedure. **Introduce about 2 g. of Permutit powder into a 100-ml. volumetric flask. Add about 5 ml. of water (no more), followed by 2 ml. of urine,**[49]

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[48] Folin and Bell: *J. Biol. Chem.*, **29**, 329 (1917). Permutit is a synthetic aluminum silicate which has the property of taking up ammonia quantitatively in neutral or weakly acid solution and of releasing it in strongly alkaline solution. The 60–80 mesh powder should be used. A suspension in water should settle quickly leaving the supernatant fluid clear. After use, it may be regenerated for further use if it is washed with water, 2 per cent acetic acid, and finally with water again.

[49] With urines very low in ammonia it may be necessary to use more urine (5 ml.) but so far as it is practicable it is better not to use more than 2 ml. and to employ a weaker

accurately measured. Rinse down the added urine by means of a little water (1 to 5 ml.), and shake gently but continuously for 5 minutes. Rinse the powder to the bottom of the flask by the addition of water (25 to 40 ml.) and decant. Add water once more and decant. (In the case of urines rich in bile it is advisable to wash once or twice more.) Add a little water to the powder, introduce 2 ml. of 10 per cent sodium hydroxide, shake for a few moments and set aside, while preparing the standard ammonium sulfate solution as follows:

Transfer 10 ml. of the standard ammonium sulfate solution (see p. 877) containing 1 mg. of nitrogen (or 5 ml. containing 0.5 mg. of nitrogen if a half-strength standard is desired) to another 100-ml. volumetric flask and add 2 ml. of 10 per cent sodium hydroxide (to balance the alkali added to the Permutit mixture in the other flask). Dilute to about 75 ml. and mix. Transfer 10 ml. of Nessler's solution (see p. 877) to a measuring cylinder. Now give the volumetric flask a vigorous whirl so as to set the solution spinning within the flask and add at once the whole of the Nessler solution in the cylinder. With another whirling movement complete the mixing of the contents of the flask. If the process of nesslerization has been successful a deep-red but crystal-clear solution is obtained. If it is not perfectly clear discard it and prepare a fresh standard. Then dilute the contents of the flask containing the Permutit and the urinary ammonia to about 75 ml., whirl the mixture, and add the Nessler reagent (10 ml.) exactly as in the case of the standard solution. Dilute the contents of both flasks to volume (100 ml.) and compare in a colorimeter with the standard set at 20 mm., or determine the densities of unknown and standard in a photometer at 480 mμ. For photometric measurement, set the photometer to zero density with a blank prepared by treating about 75 ml. of water plus 2 ml. of 10 per cent sodium hydroxide in a 100-ml. volumetric flask with 10 ml. of Nessler solution as described, diluting to 100 ml., and mixing.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \text{mg. N in Standard} = \text{mg. NH}_3\text{ N in volume of urine used}$$

Divide the result of the above calculation by the volume of urine used to obtain the ammonia-nitrogen content of the urine, in mg. per ml. (or g. per liter). From this, and the volume of the 24-hour specimen, the daily output of urinary ammonia is readily obtained.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times \text{mg. N in Standard} = \text{mg. NH}_3\text{ N in volume of urine used}$$

Further calculation is the same as for colorimetric measurement. The spectrophotometric characteristics of the Nessler color, and the conditions for accurate photometric measurement, are similar to those given on p. 878 in connection with the Folin-Farmer micro-Kjeldahl method, since similar amounts of nitrogen are dealt with here, under equivalent conditions.

Interpretation. See p. 890.

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standard (0.5 mg. instead of 1 mg. of nitrogen) for colorimetric comparison. If there is doubt about which standard to use, it is sound analytical practice to prepare both and to match the unknown against the most suitable standard as determined by inspection.

AMINO ACID NITROGEN

1. Ninhydrin Method of Van Slyke, MacFadyen, and Hamilton:[50] Principle. The urine sample, previously freed from urea by treatment with urease, is heated at 100° C. in a closed reaction vessel with ninhydrin (see p. 129). Amino acids present yield carbon dioxide quantitatively under these conditions. The carbon dioxide produced is transferred to the chamber of the Van Slyke-Neill manometric apparatus and there determined. From the amount of carbon dioxide found, the α-amino nitrogen content of the sample is readily obtained, since all of the common amino acids yield 1 mole of carbon dioxide (except aspartic acid, which yields 2 moles) per mole of α-amino nitrogen present. Proteins, peptones, peptides (other than glutathione, which has a free α-amino group), and substances other than amino acids do not react significantly. This procedure is considered to be the most specific yet devised for free amino acids.

Procedure: Removal of Urea and Preformed CO_2. **Place 2 ml. of fresh urine (or urine preserved by saturation with thymol and storage in the cold) in the all-glass reaction vessel[51] (Fig. 243) and add 1 drop of 0.04 per cent bromothymol blue.[52] If a blue color is obtained, add 1 N sulfuric acid drop by drop until color turns yellow, and then 1 N sodium hydroxide until just blue. If a yellow color is obtained initially, add 1 N sodium hydroxide until just blue. Then add 175 mg. of dry phosphate buffer mixture,[53] 0.2 ml. of a 1 per cent solution of urease,[54] and a crystal of thymol. Stopper loosely to prevent loss of water but not of carbon dioxide and incubate overnight at 37° to 40° C.**

After incubation add 1 drop of 0.04 per cent bromocresol green and a drop of caprylic alcohol. Cautiously add 5 N sulfuric acid drop by drop, with gentle whirling to minimize foaming, until the solution is just yellow (pH about 3), then add 100 mg. of dry citrate buffer mixture.[55] Add several pieces of alundum to prevent bumping and heat to boiling over a microburner.

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[50] Van Slyke, MacFadyen, and Hamilton: *J. Biol. Chem.*, **150**, 251 (1943). See also Hamilton and Van Slyke: *J. Biol. Chem.*, **150**, 231 (1943); Van Slyke, Dillon, MacFadyen, and Hamilton: *ibid.*, **141**, 627 (1941); Van Slyke and Folch: *ibid.*, **136**, 509 (1940).

[51] Obtainable from E. Machlett and Son, New York, N.Y.

[52] See Chapter 1 for the preparation of indicator solutions.

[53] *Dry Phosphate Buffer Mixture.* Grind separately in a mortar 3 parts by weight of anhydrous monopotassium phosphate and 1 part of anhydrous disodium phosphate (or 2.5 parts of disodium phosphate containing 12 molecules of water). Mix the two ground solids and grind intimately together. Dispense with a glass spoon calibrated to deliver 175 ± 15 mg. of the powder. This mixture produces a pH of 6.2 when in solution.

[54] *Urease Solution.* This is made from urease powder, prepared as described on p. 883. Dissolve the powder in water in the proportion of 1 g. to 10 ml. of water. Dilute this 10 per cent solution 1:10 with water before use to make the 1 per cent solution required. This solution contains sufficient amino acid to produce a correction (as established by the blank analysis described in the procedure) equal to several per cent of the usual amino acid content of the urine. To decrease this correction to about one-third of its initial value, wash the dry urease powder with alcohol as follows: Grind 5 g. of the urease powder in a mortar with 10 successive portions of 50 ml. each of 80 per cent ethyl alcohol. Dry the solid residue (about 2.5 g.) at room temperature *in vacuo*, and use this purified material for the preparation of the 10 per cent solution. To free the urease completely from amino acids, the dialysis purification procedure of Archibald and Hamilton (*J. Biol. Chem.*, **150**, 155 (1943)) may be used. The activity of the urease powder should be established as described by Van Slyke: *J. Biol. Chem.*, **73**, 695 (1927). (See also Van Slyke, MacFadyen, and Hamilton: *loc. cit.*) If the urease splits less than 0.1 its weight of urea per minute, a proportionately stronger solution than 1 per cent should be used.

[55] *Dry Citrate Buffer Mixture.* Grind separately in a mortar 2.06 g. of sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) and 19.15 g. of citric acid ($C_6H_8O_7 \cdot H_2O$). Mix the two ground solids and grind intimately together. Dispense with a glass spoon calibrated to deliver 100 ± 10 mg. of the powder. This mixture produces a pH of 2.5 in solution.

Boil exactly 1 minute (no longer). Cool to below 25° C. (placing in ice water for three minutes is satisfactory). Attach the short rubber connector[56] to the side arm (Fig. 243), and wipe off any water from the inside ground-glass surface of the vessel top.

Treatment with Ninhydrin. Add 100 mg. of ninhydrin[57] to the cooled reaction mixture with the aid of a small funnel having a short stem. Have the glass stopper ready with a thin film of special lubricant[58] spread over the ground-glass surface, and as soon as the ninhydrin has been added, remove the funnel and set the stopper in place, with the hole in line with the side arm, as shown in Fig. 243. Immediately attach the side arm to a good source of vacuum (preferably with attached manometer) and evacuate quickly to 30 mm. or less pressure, shaking gently if necessary to minimize effervescence. When the vessel is evacuated (10 seconds may be sufficient time), turn the stopper through 180° to close off the vessel. Press the rubber connector flat with the fingers, detach vessel and connector from the vacuum line, and insert a glass plug moistened with water or glycerol into the connector, trapping as little air as possible in the process. Tighten the stopper in place—it may be held more securely by a heavy rubber band linking stopper and side arm. Place the closed vessel upright in a wire rack and immerse completely in vigorously boiling water for 8 minutes. Remove, tighten the stopper again by a slight twist if necessary, and allow to cool to room temperature. The closed vessel may be kept for several days at this point if necessary, and if many analyses are to be done they may all be brought to this stage before proceeding.

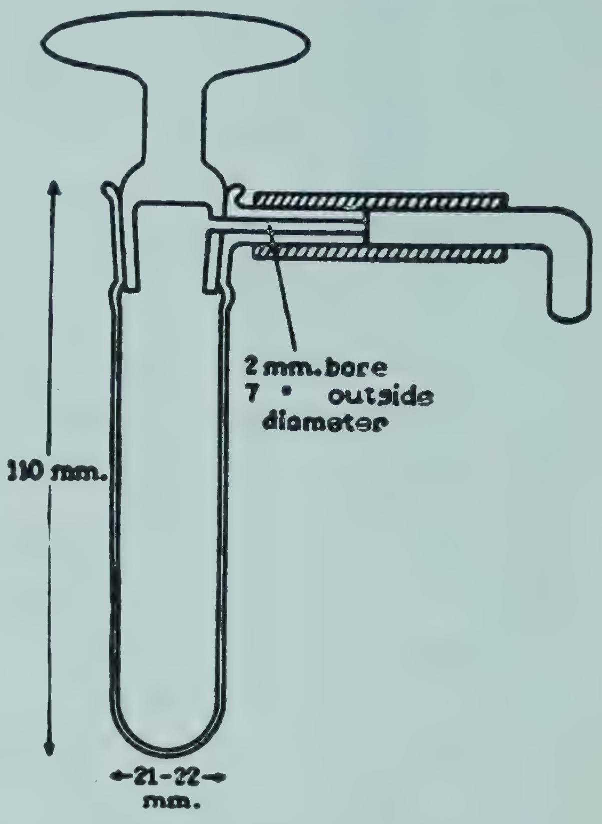

FIG. 243. REACTION VESSEL FOR NINHYDRIN AMINO ACID METHOD.

Courtesy, Hamilton and Van Slyke: *J. Biol. Chem.*, **150**, 231 (1943).

Absorption and Measurement of CO_2. Warm the contents of the reaction vessel to 38° to 40° C. by immersion in a beaker of water at this temperature for 10 minutes. While waiting, deliver 2 ml. of the carbonate-free 0.5 N sodium hydroxide in sodium chloride solution[59] from the storage container

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[56] A 5-cm. length of stethoscope tubing is used (3 mm. bore, 12 mm. outside diameter). Before using for the first time, clean the tubes inside with a test-tube brush, immerse in acidified water in a round bottomed flask, and boil for 30 minutes. Remove the flame, stopper the flask, and cool under the tap. The vacuum caused by cooling draws excess gases from the rubber. When bubbles no longer appear, open the flask and wash the tubes with water. One treatment suffices.

[57] Dispense with a glass spoon calibrated to deliver approximately the required amount (within 10 per cent).

[58] A lubricant which does not leak at high temperatures must be used. "Nevastane XX heavy lubricating grease," obtainable from E. Machlett and Son, New York, is satisfactory. A suitable lubricant may be prepared in the laboratory as follows: Mix 35 g. of aluminum distearate to a paste in 100 ml. of heavy paraffin mineral oil. Heat with continuous stirring in a beaker over a low flame to effect solution of the soap. Allow to cool, then work up the friable gel to a smooth translucent paste on a glass plate with a steel spatula, preferably with warming to 45° to 50° C.

[59] Dissolve solid sodium hydroxide in an equal weight of water and allow to stand until

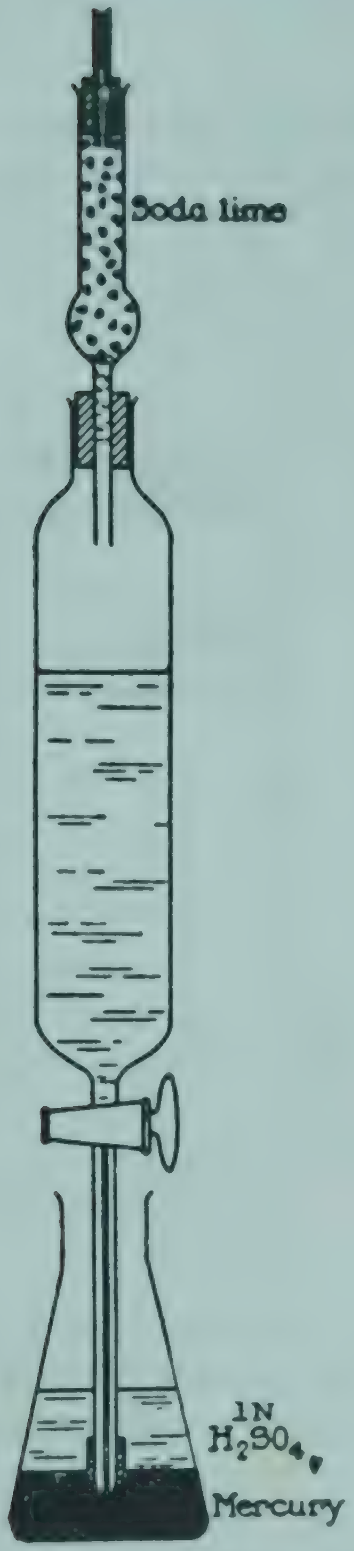

FIG. 244. STORAGE CONTAINER FOR ALKALINE SOLUTIONS, HUNG WITH TIP PROTECTED FROM ATMOSPHERIC CARBON DIOXIDE.

Courtesy, Van Slyke and Folch: *J. Biol. Chem.*, **136**, 509 (1940).

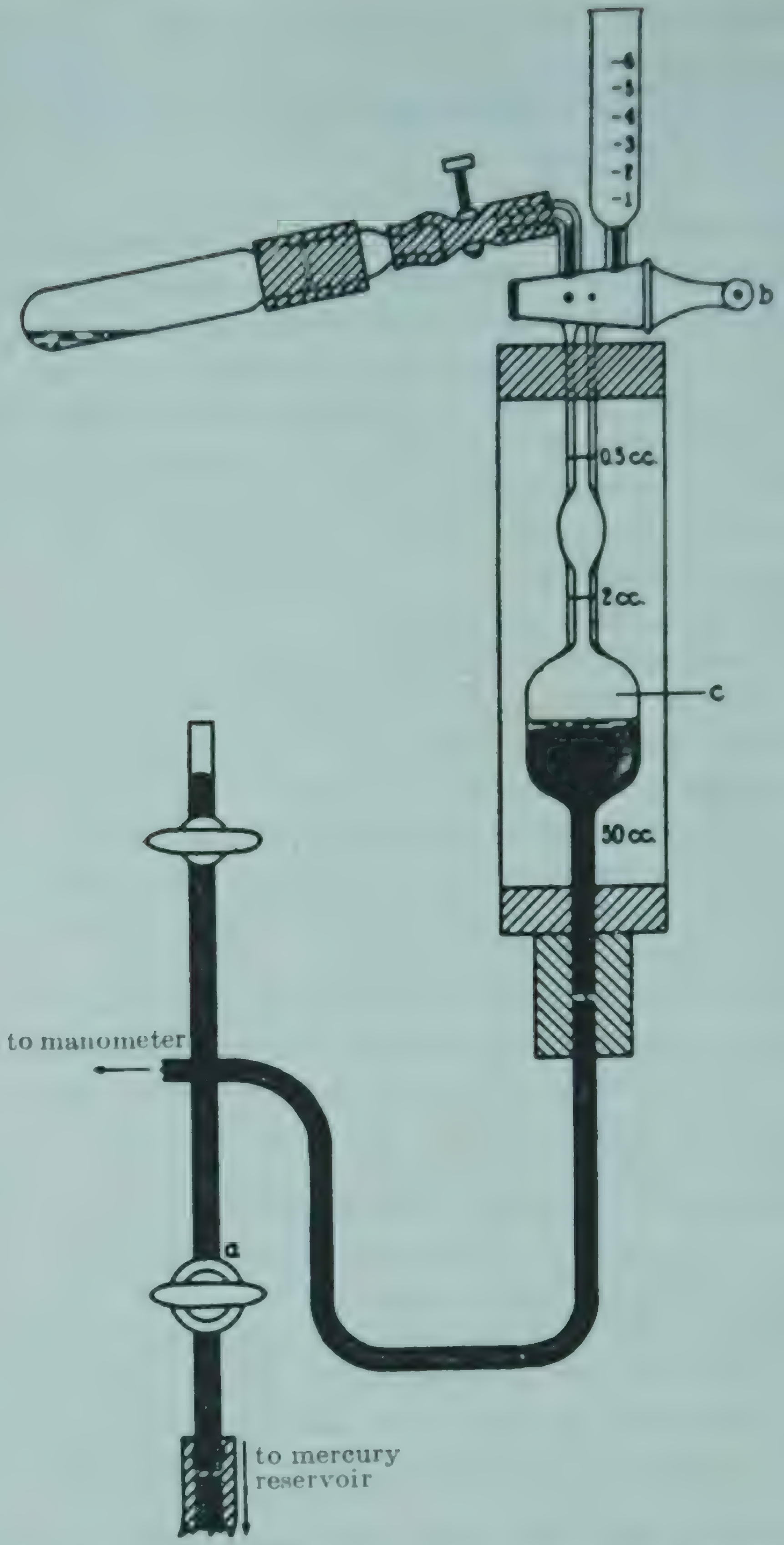

FIG. 245. MANOMETRIC CHAMBER WITH REACTION VESSEL (EARLIER TYPE) ATTACHED BUT NOT YET CONNECTED WITH INTERIOR OF CHAMBER.

Courtesy, Van Slyke, Dillon, MacFadyen, and Hamilton: *J. Biol. Chem.*, **141**, 627 (1941).

the carbonate settles. Standardize the supernatant solution by pipeting 7 ml. into water and titrating with standard (2 N or stronger) acid. From the result, determine how much of the 1:1 NaOH is required to make 250 ml. of 0.5 N solution. Fill a 250-ml. volumetric flask to within about 10 ml. of the mark with concentrated CO_2-free NaCl solution (dissolve 250 g. of NaCl in 750 ml. of water, deaerate and store as described on p. 715). Pipet the correct amount of 1:1 NaOH solution into the flask, delivering it below the surface of the salt solution. Add a few drops of 1 per cent alizarin solution as indicator, fill to the mark with salt solution, and mix. Transfer to a storage container (Fig. 244 in text) to protect from atmospheric carbon dioxide.

equipped with a rubber tip (Fig. 244), into the chamber of the Van Slyke-Neill apparatus (Fig. 188), through a mercury seal in the cup (see Fig. 190 and p. 711 for method of delivery). Seal the cup capillary with a little mercury, lower the mercury in the chamber to about the middle of the chamber, and close the cock leading to the leveling bulb (cock *a* in Fig. 245). By this time the reaction-vessel contents should be sufficiently warm. Remove the vessel from the water bath, remove the glass plug from the side arm, attach the rubber connector to a source of vacuum, and reevacuate the side arm for a few seconds. Pinch the rubber connector flat with the fingers, detach from the vacuum line, and at once attach to the side arm of the Van Slyke-Neill apparatus. Fig. 245 shows the conditions prevailing at this point. Turn the stopcock of the reaction vessel and cock *b* of the chamber so as to connect vessel and chamber and permit gas to pass from one to the other. Transfer the CO_2 in the reaction vessel to the chamber (where it is absorbed by the alkali present) by repeatedly raising and lowering the mercury in the chamber by manipulation of the leveling bulb and cock *a*. The first lowering of the mercury must be done carefully to prevent bumping in the reaction vessel, and at each lowering the reaction vessel is shaken by hand to distribute the fluid along the walls. Five excursions of the mercury suffice to transfer all the CO_2, each excursion taking about 10 seconds. After the last upward excursion, lower the mercury in the chamber to about the middle point, and close cocks *a* and *b*. Remove the reaction vessel and fill the side arm of the cock capillary of the manometric apparatus with mercury by suction from a small bottle containing mercury (see Fig. 246).

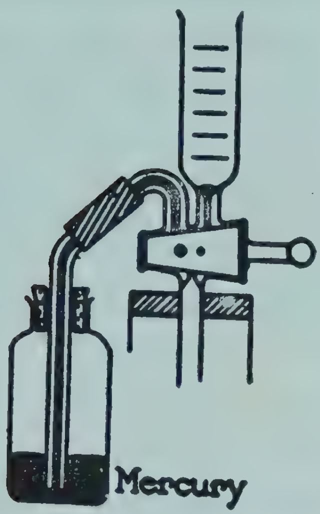

FIG. 246. SMALL BOTTLE OF MERCURY ARRANGED FOR SEALING CAPILLARY OF MANOMETER STOPCOCK.

Courtesy, Van Slyke and Folch: *J. Biol. Chem.*, **136**, 509 (1940).

Open cock *a*, raise the leveling bulb to about even with cock *b*, then close *a*. Open *b* carefully to the right-hand cup at the top, allow the gases present to escape through the cup, then admit mercury to the chamber through cock *a* until the fluid in the chamber just reaches the top of the chamber, at cock *b*. Close *a* and then *b*. Place the leveling bulb at its normal position (approximately even with the 50-ml. mark at the bottom of the chamber), place a little mercury in the cup, and seal the cock capillary with mercury in the usual way.

Through the mercury seal, add exactly 1 ml. of 2 N lactic acid in sodium chloride solution[60] from a stopcock pipet provided with a rubber ring on the tip, as illustrated in Fig. 190, p. 711, then seal the cock with a little mercury. By manipulation of the leveling bulb and cock *a*, lower the mercury in the chamber to the 50-ml. mark, close *a*, and shake the chamber for 20 to 30 seconds. The liberated CO_2 drives the mercury below the 50-ml. mark Raise the leveling bulb, open *a* carefully, and adjust the mercury level to the 50-ml. mark. Close *a* and shake the chamber for 3 minutes. Place the leveling bulb high, open *a*, and admit mercury into the chamber until the level of the *fluid* (not mercury) meniscus is exactly at the 2-ml. mark on the chamber. This adjustment must be done in a consistent manner. Allow the mercury to enter through *a* steadily, without jerky oscillation of the chamber contents,

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[60] Dilute 1 volume of concentrated lactic acid (sp. gr. 1.20) with 4 volumes of the concentrated NaCl solution described in the previous footnote.

and complete the adjustment in 30 to 40 seconds. If the adjustment is missed the first time (or for a duplicate reading) lower the mercury to the 50-ml. mark, shake for one minute, and repeat. With the fluid meniscus at the 2-ml. mark, read the manometer. This reading is p_1.

Replace the leveling bulb to the normal position, open cock *a*, and place 0.5 ml. of 5 N sodium hydroxide solution[61] in the cup above cock *b*. Admit the alkali to the chamber slowly, over a period of about half a minute, until only enough is left to fill the capillary beneath the cup. Add a little water and mercury to the cup, and seal the cock with mercury. If the viscous alkali forms a solid column in the top of the chamber, it may be broken up by admitting a little mercury in short bursts. Mix the alkali and ensure complete absorption of CO_2 by raising and lowering the mercury in the chamber 3 times in short excursions, then bring the fluid meniscus to just below the 2-ml. mark on the chamber. Allow 1 minute for drainage, then adjust exactly to the 2-ml. mark. Read the manometer; this reading is p_2.

CALCULATION. The pressure P_{CO_2} due to the carboxyl carbon dioxide of the amino acids present is found as follows:

$$P_{CO_2} = p_1 - p_2 - c$$

where *c* is the correction due to the blank. To establish *c*, run through an entire analysis as described above (including incubation with urease, etc.) on 2 ml. of water instead of urine. The difference between the p_1 and p_2 readings for the water blank analysis equals *c*. This blank will serve to correct for small amounts of amino acids which may be present in the urease preparation, as well as for certain possible variables in the manometric measurement itself.

To obtain the amino acid nitrogen content of the sample, multiply P_{CO_2} by the proper factor, obtained from the accompanying table.

$$P_{CO_2} \times \text{factor} = \text{mg. } \alpha\text{-amino nitrogen per liter of urine}$$

The temperature column in the table refers to the temperature of the water in the jacket surrounding the chamber at the time the p_1 and p_2 readings are made, and *a* is the volume of the gas phase when the manometer is read; *a* is ordinarily 2 ml., but may be reduced to 0.5 ml. if only small amounts of amino acids are present.

FACTORS FOR USE IN CALCULATING MG. OF α-AMINO NITROGEN PER LITER OF URINE
(SAMPLE VOLUME = 2 ML.)

Temperature ° *C.*	$a = 2.0$	$a = 0.5$	*Temperature* ° *C.*	$a = 2.0$	$a = 0.5$
15	0.802	0.2010	25	0.770	0.1930
16	0.798	0.2002	26	0.767	0.1923
17	0.795	0.1994	27	0.764	0.1916
18	0.792	0.1985	28	0.762	0.1908
19	0.789	0.1977	29	0.758	0.1901
20	0.786	0.1969	30	0.756	0.1894
21	0.782	0.1961	31	0.752	0.1886
22	0.780	0.1953	32	0.750	0.1879
23	0.776	0.1945	33	0.747	0.1872
24	0.773	0.1938	34	0.744	0.1866

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[61] Mix 1 volume of 1:1 NaOH solution with 3 volumes of water.

Interpretation. The α-amino acid nitrogen content of normal urine appears to make up about 1 per cent (or possibly even less) of the total urinary nitrogen, corresponding to a daily excretion of 100 to 150 mg. of α-amino nitrogen. Results by other methods, e.g., the nitrous acid method of Van Slyke and Kirk,[62] the formol titration procedure (see following method), and the copper titration procedure of Albanese and Irby,[63] tend to be either slightly or significantly higher than results by the ninhydrin procedure described here, presumably because of differences in specificity for α-amino nitrogen. Little is known concerning the significance of normal or pathological variation in amount of urinary α-amino nitrogen. Excretion may be largely increased in disorders associated with tissue waste as typhoid, in pronounced atrophy of the liver, acidosis, etc.

Microbiological assays for individual free amino acids in human urine show a preponderance of histidine, cystine, and glycine, average daily excretions being 150 to 250, 60 to 110, and 120 mg., respectively. Threonine, 50 to 60 mg., and lesser amounts of other amino acids are also found.[64] Glycine, glutamic and aspartic acids, and lysine are also found in conjugated form.

2. *Henriques-Sørensen Formol Titration Method:*[65] *Principle.* A solution containing amino acids is nearly neutral in reaction. If formaldehyde be added, however, a reaction takes place with the formation of derivatives which are more strongly acid in reaction due to the destruction of the basic properties of the amino groups (see p. 130 for discussion and equations). The carboxyl groups may then be titrated using phenolphthalein as an indicator. The acidity as shown by the titration is a measure of the amount of amino acid nitrogen present. Ammonia likewise reacts with formaldehyde in a similar manner as is shown in the following equation:

$$4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 6H_2O + 4HCl$$

Hence the formol titration in the presence of ammonia gives results which include both amino acid and ammonia nitrogen. Ammonia may be determined and a correction applied, or the ammonia may be removed by means of phosphotungstic acid. Phosphates also interfere by obscuring the end point and are removed by the addition of barium salts. Polypeptides and still more complex protein derivatives likewise react with formol to a certain degree so that the results do not strictly represent "amino acid nitrogen."

The method is, with some modifications involving the preparation of the solution to be titrated, applicable in the determination of amino acids in any medium, e.g., urine, protein digests,[66] etc. The presence of buffers or poorly dissociated acids, e.g., some fatty acids, will tend to give values which are too high. Certain of the amino acids when present in large amounts will give erroneous results, but in the ordinary urine or digest these errors are either negligible or compensate each other. In the titration of colored solutions the control solution which is necessary in this method must be colored to correspond with the color of the unknown solution. The accuracy of the formol titration is considerably improved by controlling the final pH adjustment and titration potentiometrically.

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[62] Van Slyke and Kirk: *J. Biol. Chem.*, **102**, 651 (1933).

[63] Albanese and Irby: *J. Biol. Chem.*, **153**, 583 (1944).

[64] Dunn, Camien, Shankman, and Block: *Arch. Biochem.*, **13**, 207 (1947); Gutman and Alexander: *J. Biol. Chem.*, **168**, 527 (1947); Christensen, Cooper, Johnson, and Lynch: *ibid.*, **168**, 191 (1947); Sheffner, Kirsner, and Palmer: *ibid.*, **175**, 107 (1948).

[65] Henriques and Sørensen: *Z. physiol. Chem.*, **64**, 120 (1909). See also Northrop: *J. Gen. Physiol.*, **9**, 767 (1926).

[66] Northrop (*J. Gen. Physiol.*, **9**, 767 (1926)) has improved the formol titration by titrating first to pH 7 with neutral red as the indicator, then adding formaldehyde and titrating to pH 9 with phenolphthalein.

Procedure. The solution to be analyzed, if carbonates, phosphates, and ammonia are absent, is made neutral to litmus (paper) and the solution titrated with formaldehyde as below.[67] In case carbonates, phosphates, or ammonia are present a preliminary treatment is necessary which will vary according to the quantity of ammonia present.

(a) FOR SMALL AMOUNTS OF AMMONIA. **Applicable to most urines. Fifty ml. of the material under examination are pipeted into a 100-ml. measuring flask and 1 ml. of phenolphthalein solution[68] and 2 g. of solid barium chloride are added; the whole is shaken, to saturate the solution with barium chloride; saturated barium hydroxide solution is added until the red color of the phenolphthalein develops and then an excess of 5 ml. is added. The flask is filled to the graduation mark with water, shaken and permitted to stand for 15 minutes, after which it is filtered through a dry filter. Place 80 ml. of the clear red filtrate (which corresponds to 40 ml. of the liquid under examination) in a 100-ml. measuring flask, neutralize to litmus and dilute to 100 ml. with freshly boiled water. Equal portions of this solution, 40 ml. (equivalent to 16 ml. of the original solution), may be taken for analysis, one for the formol titration and the other for the determination of ammonia nitrogen.[69]**

(b) FOR LARGE AMOUNTS OF AMMONIA. **After the treatment with phenolphthalein, barium chloride, and barium hydroxide, and after the solution has been diluted to 100 ml. as in (a) above, the ammonia is distilled off, _in vacuo_.[70]**

In case the solution is deeply colored, as in protein digests, it may be necessary to decolorize[71] before the titration is attempted.

Final Titration. For the final titration a volume of from 20 to 40 ml. which contains approximately 25 mg. of nitrogen is the most desirable. A control solution is run composed of an equal volume of boiled distilled water and 20 ml. of the formaldehyde mixture.[72] This control solution is colored[73] so that its tint matches that of the solution to be titrated.

To this control is added about half the volume of 0.2 N alkali which will be used in the titration of the solution under investigation and it is then titrated with 0.2 N acid to a faint red (first stage).[74]

An additional drop of 0.2 N alkali is added, which imparts a distinct red to the solution (second stage).

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[67] As a standard of comparison the litmus paper used for neutralization is contrasted with a similar piece dipped in a phosphate solution having a neutral reaction (M/15 KH_2PO_4 and M/15 Na_2HPO_4 in the proportion 40:60).

[68] A solution of 0.5 g. of phenolphthalein in 50 ml. of alcohol and 50 ml. of water.

[69] The determination of ammonia may be dispensed with in case a separate determination is made.

[70] For particulars with regard to the distillation, etc., see Henriques and Sorensen: *Z. physiol. Chem.*, **64**, 137 (1909).

[71] For methods see Jessen-Hansen: *Abderhalden's Arbeitsmethoden*, Vol. 6, p. 262, 1912.

[72] The formaldehyde solution is freshly prepared for each set of determinations as follows: to 50 ml. of commercial formaldehyde (formol) (30 to 40 per cent) add 1 ml. of the phenolphthalein solution. 0.2 N alkali is then added until the mixture acquires a faint red color. The volume of the formaldehyde used will vary with the volume of the solution to be analyzed; approximately 10 ml. of the formalin solution are added for each 20 ml. of the unknown solution.

[73] Solution of Bismarck brown is very satisfactory for urines. Tropeolin O, tropeolin OO, *p*-nitrophenol, methyl orange, or alizarin sulfonate may be used.

[74] This procedure is recommended in order that the final volume of the control and the unknown solutions shall be approximately the same when the process is complete.

The solution to be analyzed is now titrated to the color produced in the second stage of the control. The formaldehyde mixture is now added; 10 ml. for each 20 ml. of the solution, and the mixture again titrated to the second stage with 0.2 N alkali.[75]

Two drops of the 0.2 N alkali are now added to the control solution which assumes a deep red color (third stage). 0.2 N alkali is now added to the solution under examination until it assumes a color corresponding to the third stage of the control. This completes the titration.

CALCULATION. The calculations are similar to those which pertain to any acidimetry procedure. Each ml. of an 0.2 N alkali or acid solution is equivalent to 0.0028 g. of nitrogen. An example will illustrate the procedure: 40 ml. of solution (16 ml. of urine) required 5.10 ml. of 0.2 N NaOH; control, 0.10 ml. of 0.2 N NaOH; total required for amino acids 5.00 ml. equivalent to 0.014 g. of nitrogen. Ammonia nitrogen in 16 ml. of urine, 0.007 g. of N. Then $0.014 - 0.007 = 0.007$ g. amino acid nitrogen in 16 ml. of urine.

Interpretation. The excretion of total amino acid nitrogen by a normal adult, as determined by this procedure, averages between 0.4 to 1.0 g. per day or from 2 to 6 per cent of the total nitrogen. Free amino acid nitrogen (see previous procedure) is considerably less than this, ordinarily 0.5 to 1.0 per cent of the total nitrogen. This discrepancy is due to the relative nonspecificity of the formol titration. For further aspects of interpretation, see preliminary discussion, and also previous procedure.

3. Other Methods. Folin proposed the colorimetric determination of amino acids in urine by reaction with naphthoquinone sulfonic acid,[76] after preliminary treatment of the urine with Permutit to remove ammonia, which interferes. Although this color reaction is sufficiently reliable for its use in the determination of blood amino acids where interference by ammonia does not arise, the procedure is not recommended for urine analysis since the Permutit treatment removes some of the amino acids as well as ammonia. A more satisfactory procedure for removal of ammonia might render the method applicable to urine. Albanese and Irby[77] have proposed a simple titrimetric method for the determination of urinary amino acid nitrogen; their procedure appears to have approximately the specificity of the formol titration. For details of the nitrous acid reaction for urinary amino acids, see Van Slyke and Kirk.[78]

CREATININE

Folin's Method: Principle. This method is based upon the characteristic property possessed by creatinine, of yielding a certain definite color reaction in the presence of picric acid in alkaline solution. This reaction (Jaffé reaction) is due to the formation of a red tautomer of creatinine picrate.[79] The production of this tautomer is "dependent upon the formation of a salt, a keto-enol change within the creatinine molecule, and a change in the picric acid molecule involving the hydrogens in the *meta* positions and, probably all three nitro groups."[80]

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[75] This is best accomplished by adding alkali until the color is deeper than that of the control, then acid again until lighter, and finally alkali to the desired color.

[76] This procedure is described for amino acids in blood. See p. 565.

[77] Albanese and Irby: *J. Biol. Chem.*, **153**, 583 (1944).

[78] Van Slyke and Kirk: *J. Biol. Chem.*, **102**, 651 (1933).

[79] Greenwald and Gross: *J. Biol. Chem.*, **59**, 601 (1924).

[80] Greenwald: *J. Am. Chem. Soc.*, **47**, 1443 (1925); *J. Biol. Chem.*, **80**, 103 (1928).

In the original Folin macromethod,[81] 10 ml. of urine are used for a determination, the color is developed at a final volume of 500 ml., and comparison is made visually against an artificial standard (0.5 N potassium bichromate solution) under rigorously defined conditions. The Folin micro modification described here is preferred because it requires less urine, is based upon comparison against standard creatinine solutions, and is therefore more flexible and accurate, and is adaptable to either colorimetric or photometric measurement.[82]

Procedure.

(a) FOR COLORIMETRIC COMPARISON. **Measure 1 ml. of urine into a 100-ml. volumetric flask, and in a second similar flask place 1 ml. of the standard creatinine solution,[83] containing 1 mg. of creatinine per ml. To each flask add 20 ml. of 1 per cent picric acid solution[84] (measured with sufficient accuracy from a graduated cylinder), followed by 1.5 ml. of 10 per cent sodium hydroxide solution. Mix gently and allow to stand 15 minutes. Dilute to the mark with water and mix by inversion. Compare standard against unknown in the usual way, setting the standard at 20 mm.**

(b) FOR PHOTOMETRIC MEASUREMENT. **Measure 0.5 ml. of urine into a 100-ml. volumetric flask and add 0.5 ml. of water. In a second flask, which serves as a blank, place 1 ml. of water. To each flask add 20 ml. of 1 per cent picric acid solution, followed by 1.5 ml. of 10 per cent sodium hydroxide solution. Mix gently and allow to stand 15 minutes. Dilute to the mark with water and mix by inversion. Transfer portions of blank and unknown to photometer cuvettes. Set the photometer to zero density with the blank, at 520 mμ, and determine the density of the unknown. Alternately, the photometer may be set to zero density with water, and the densities of both blank and unknown determined. The density of the blank is then subtracted from the measured density of the unknown, to obtain the true density of the unknown.**

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \begin{matrix}\text{mg. creatinine}\\ \text{in Standard}\end{matrix} = \begin{matrix}\text{mg. creatinine in volume}\\ \text{of urine analyzed}\end{matrix}$$

With a urine volume of 1 ml., and a 1-mg. standard, the result of the calculation gives the creatinine content of the urine directly, in mg. per ml., or g. per liter. Urines containing 0.75 to 1.25 g. of creatinine per liter (i.e., the standard and unknown read within a few mm. of each other) may be accurately determined with the 1-mg. standard described. For values outside this range, the calculation is not accurate because of the deviation from Beer's law shown by the Jaffé reaction for creatinine. In such cases the determination must be repeated using less or more urine as the case may be, to provide a portion of sample containing approximately 1 mg. of creatinine. When this is done, differences in volume between standard and unknown *before adding the*

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[81] Folin: *J. Biol. Chem.*, **17**, 469 (1914).

[82] For studies on the photometric determination of creatinine by the Jaffé reaction, see Bonsnes and Taussky: *J. Biol. Chem.*, **158**, 581 (1945); Peters: *J. Biol. Chem.*, **146**, 179 (1942). The use of 3,5-dinitrobenzoic acid for the colorimetric determination of creatinine has been proposed by Benedict and Behre: *J. Biol. Chem.*, **114**, 515 (1936), and Langley and Evans: *ibid.*, **115**, 333 (1936).

[83] *Standard Creatinine Solution:* Dissolve 1 g. of pure dry creatinine in sufficient 0.1 N hydrochloric acid to make 1 liter, and mix well. This solution contains 1 mg. of creatinine per ml., and is stable indefinitely. Pure creatinine may be purchased from laboratory supply houses, or may be made from urine by Benedict's method (see p. 800). Creatinine-zinc chloride may be used in place of creatinine, in which case 1.61 g. of the creatinine-zinc chloride salt is used per liter of 0.1 N acid to give the standard solution containing 1 mg. of creatinine per ml.

[84] Prepared from purified picric acid (see Appendix). Keep in a dark bottle.

picric acid must be equalized by the proper addition of water to one or the other as required. The further procedure and the calculations are as described. For very dilute urines (creatinine content less than 0.2 g. per liter), the Shaffer modification of the Folin procedure may be used.[85]

For photometric measurement: the creatinine content of the unknown is established from its photometric density by reference to a calibration curve showing the densities for known amounts of creatinine determined by the procedure described. Direct calculation of results in terms of Beer's law is not valid in this procedure because of the lack of adherence to Beer's law over the concentration range in question.

To prepare a calibration curve, measure 0.0, 0.25, 0.50, 0.75, and 1.00 ml. portions of standard creatinine solution, containing 1 mg. per ml., into separate 100-ml. volumetric flasks (preferably in duplicate). Add water, where necessary, to bring each to 1 ml. volume, and treat with picric acid and alkali exactly as described under "Procedure for Photometric Measurement," above. Determine the densities for each of the standards, using the first or blank solution to set the photometer to zero density, or set the photometer to zero density with water and subtract the determined density of the blank from that for each standard to obtain the true densities of the standards. On a sheet of ordinary cross-section paper, plot the density of each standard on the y-axis against the amount of creatinine present, in mg., on the x-axis. Draw a smooth curve to include all the points. A curve similar to that shown in Fig. 247 will be obtained.

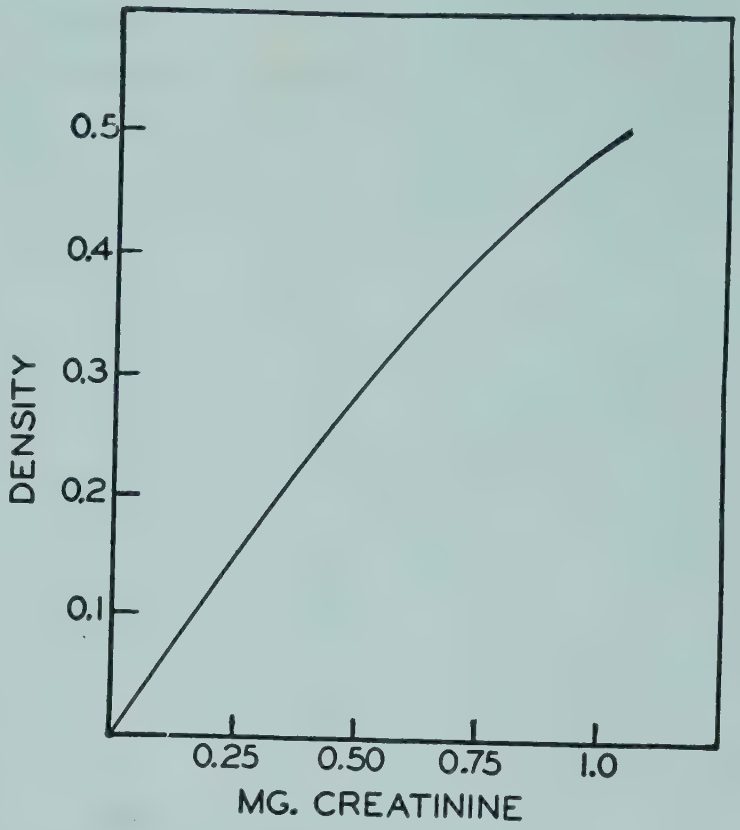

FIG. 247. TYPICAL CALIBRATION CURVE FOR PHOTOMETRIC DETERMINATION OF CREATININE IN URINE.

The amount of creatinine present in an unknown is obtained by reference to the curve. If 0.5 ml. of urine is used in an analysis, the result from the curve gives the creatinine content in this volume of sample. Multiply by 2 to obtain the creatinine content in mg. per ml. or grams per liter.

The spectrophotometric characteristics of the creatinine color are shown in Fig. 248. At 520 mμ, and in a 1-cm. cuvette, the densities for various amounts of creatinine have approximately the values indicated in Fig. 247. Any creatinine content up to about 2 g. per liter may be accurately determined under these conditions. For higher values, or for photometric measurement at greater depth of solution, the urine should be diluted, a portion of the diluted sample used for analysis, and the creatinine content as read from the curve multiplied by the dilution to obtain the true creatinine content of the original undiluted urine.

The sources of error in the use of a previously prepared calibration curve have been discussed in Chapter 23. For accurate results the curve should be checked at intervals and reconstructed if necessary. In an analysis, every effort should be made to have the conditions as nearly identical as possible with those prevailing at the time the curve was established; the time of standing (after adding the alkali and after diluting) and the room temperature are particularly important in this connection. The curve should be checked or reestablished with each new lot of picric acid.

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[85] Shaffer: *J. Biol. Chem.*, **18**, 525 (1914).

Interpretation. The daily excretion of creatinine by normal adults ranges from about 1.0 to 1.8 g.; under certain conditions it may be considerably higher.[86] The value is nearly constant from day to day for a given normal individual, being influenced by the diet only to the extent that the diet itself contains significant amounts of creatinine, as in the case of a heavy meat diet. Creatinine excretion is not influenced by exercise or by the level of nitrogen metabolism in the body. Creatinine appears to be entirely a waste product, unutilizable by the body, and is excreted almost if not entirely quantitatively when ingested or injected. The relative constancy of creatinine excretion on a creatinine-free diet appears to reflect some constant metabolic process involving the body creatine (from which creatinine is almost certainly derived), but the nature of this process is still obscure (see p. 797 for further discussion) and therefore the significance of a constant creatinine excretion cannot be too precisely evaluated. Since the bulk of the creatine of the body is in the muscles, there is a rough correlation between the creatinine excretion and the amount of muscular tissue in the body; for example, obese individuals excrete less creatinine relative to their body weight than do thin persons. The number of mg. of creatinine excreted daily per kg. of body weight is known as the *creatinine coefficient*. It has a normal range of 19 to 30. Some investigators designate the creatinine coefficient in terms of the number of mg. of creatinine-*nitrogen* excreted daily per kg. of body weight; the normal range in this case is 7 to 11.

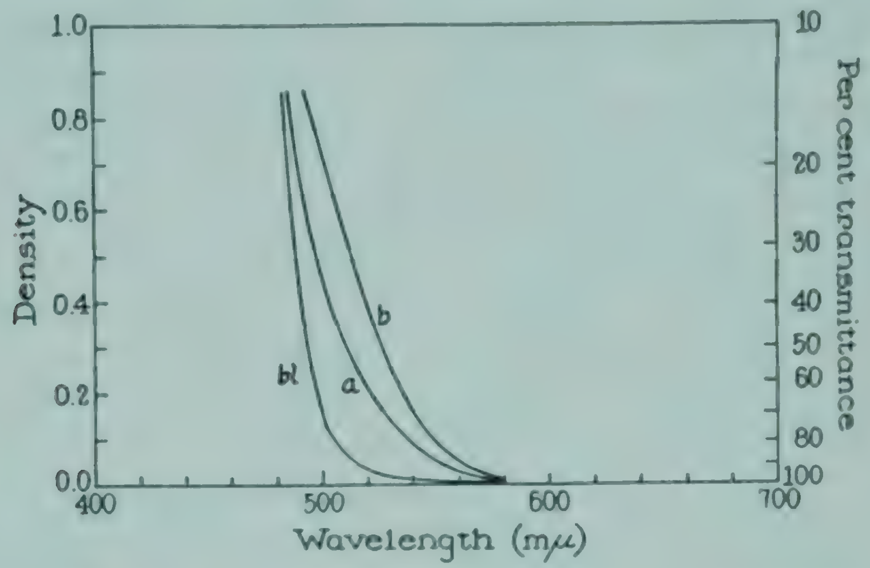

FIG. 248. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN URINARY CREATININE METHOD.

For the alkaline picrate blank (*bl*), blank plus 0.25 mg. (*a*), and 0.50 mg. (*b*) of creatinine. Solution depth, 1 cm.

Creatinine excretion is decreased in disorders associated with muscular atrophy and muscular weakness. It increases with increased tissue catabolism as in fever.

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[86] See Hobson: *Biochem. J.*, 33, 1425 (1939).

CREATINE

1. Folin Method:[87] ***Principle.*** Creatine on boiling with acid is transformed into creatinine. By determining the creatinine content before and after treatment with acid (picric acid is used here), the amount of creatine present may be obtained by difference. The method is not applicable to diabetic urines, since acetone and glucose interfere.

Procedure.[88] **Measure the urine (1 ml. for colorimetric measurement, 0.5 ml. for photometric measurement) into a 300-ml. flask. Add 20 ml. of 1 per cent picric acid solution and a few small pebbles or pieces of alundum. Weigh the flask and contents on a rough balance to the nearest gram. Add about 150 ml. of water and heat to boiling. Boil gently for 45 minutes, then more rapidly until the volume has been reduced to somewhat under the original volume of urine plus picric acid solution, as established by weighing. Add sufficient water to the flask to restore the original weight, within about a gram. Cool to room temperature (this is important). Add 1.5 ml. of 10 per cent sodium hydroxide solution, and mix. Let stand 15 minutes. Rinse into a 100-ml. volumetric flask, dilute with water to the mark, and mix. For colorimetric measurement, compare against a standard creatinine solution, containing 1 mg. of creatinine, prepared in a second 100-ml. volumetric flask as described on p. 900 for the determination of urinary creatinine. For photometric measurement, determine the density (corrected for the picrate blank) as described on p. 900, and obtain results from a calibration curve, as described below.**

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \begin{array}{c}\text{mg. creatinine}\\ \text{in Standard}\end{array} = \begin{array}{c}\text{mg. ``total'' creatinine}\\ \text{in vol. of urine used}\end{array}$$

From this, the "total" creatinine content per liter or in the 24-hour sample may be obtained. Subtract from the "total" creatinine value the preformed creatinine content, determined separately as described on p. 900, to obtain the creatine content (expressed as creatinine).

$$(\text{Total creatinine} - \text{Preformed creatinine}) = \text{Creatine (as creatinine)}$$

To convert creatine expressed as creatinine into the amount of creatine itself, multiply by 1.16:

$$(\text{Total creatinine} - \text{Preformed creatinine}) \times 1.16 = \text{Creatine}$$

The same precautions concerning close agreement between standard and unknown that are emphasized in the determination of urinary creatinine are applicable here. The total creatinine content of the sample should not be greater than about 1.25 g. per liter, when a 1-mg. standard is used. If it is higher than this, repeat the analysis using less urine, or an aliquot of diluted urine.

For photometric measurement: from the determined density of the unknown (corrected for the picrate blank), obtain the "total" creatinine content of the volume of urine used by reference to a calibration curve, as described on p. 901 for the photometric determination of urinary creatinine (the same curve may be used). Determine the creatine content as described above, by subtracting from the "total" creatinine content the amount of preformed creatinine present, determined separately. If the photometric density of the unknown is beyond the limits of the calibration curve, repeat the analysis using less urine.

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[87] Folin: *J. Biol. Chem.*, **17**, 469 (1914).

[88] The reagents required are those used for the determination of urinary creatinine (p. 900).

Interpretation. Creatine occurs only in small amount (approximate range 0 to 200 mg. per day) in the urine of normal adults, but is found in larger amounts in the urine of children. More creatine is found in the urine during activity than when at rest. Hobson[89] found significant creatinuria (in one instance 1.4 g. per day) in the case of adult male athletes in training, on a high carbohydrate diet; decrease in carbohydrate intake resulted in a markedly decreased excretion of creatine. Albanese and Wangerin[90] found significant amounts of creatine in the urine of 30 normal adults, with no differences between sexes. According to these investigators, earlier views that creatinuria is not found in normal adults are possibly due to technical difficulties in the analysis.

Increased creatine excretion is noted in pregnancy, in fasting, and after high water ingestion. A significant creatinuria is noted in many pathological conditions associated with malnutrition and disintegration of muscular tissue, etc. Ingestion of creatine by normal adults does not increase the creatine content of the urine, i.e., the ingested creatine is completely retained. In certain pathological conditions, however, notably progressive muscular dystrophy, this ability to retain ingested creatine is impaired and extra creatine appears in the urine after a test dose. This is the basis for the *creatine tolerance test*,[91] which is sometimes used clinically for diagnostic purposes.

2. Other Methods. Various modifications of the acid treatment for the conversion of creatine to creatinine have been described. In the original Folin method, the urine is heated with an equal volume of 1 N hydrochloric acid for three hours on a boiling water bath; Folin felt that the picric acid method described here is superior to this first procedure. Autoclaving with picric acid solution at 115° to 120° C. for 20 minutes has also been proposed; according to Albanese and Wangerin,[90] this does not give complete conversion, and Bonsnes and Taussky[92] have confirmed this. In the Benedict[93] procedure, the urine is taken to dryness in the presence of 1 to 2 volumes of normal hydrochloric acid, with a few granules of lead to minimize pigment formation. This procedure appears to give complete conversion, but it has not as yet been shown to be applicable to the small amounts of creatine concerned in modern micromethods. Bonsnes and Taussky[92] and Peters[94] have described procedures for the determination of urinary creatine under conditions whereby the diluted sample is treated by procedures similar to those used for blood filtrates,[95] thus permitting the determination of both blood and urine creatine and creatinine in essentially the same manner.

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[89] Hobson: *Biochem. J.*, **33**, 1425 (1939).

[90] Albanese and Wangerin: *Science*, **100**, 58 (1944). See, however, Lambert: *J. Biol. Chem.*, **161**, 679 (1945).

[91] See Milhorat and Wolff: *Arch. Neurol. Psychiat.*, **38**, 992 (1937) for discussion and review.

[92] Bonsnes and Taussky: *J. Biol. Chem.*, **158**, 581 (1945).

[93] Benedict: *J. Biol. Chem.*, **18**, 191 (1914).

[94] Peters: *J. Biol. Chem.*, **146**, 179 (1942).

[95] See Chapter 23.

URIC ACID

1. Direct Colorimetric Method of Benedict and Franke:[96] ***Principle.*** The diluted urine is treated directly with arsenophosphotungstic acid reagent and sodium cyanide. The blue color obtained is compared with that of a standard uric acid solution treated in the same way. This method is known to be somewhat nonspecific for uric acid, as are all "direct" methods (see discussion under the other methods described in this section) but is believed to be quite satisfactory for many purposes.

Procedure.[97] **The urine[98] is so diluted that 10 ml. will contain between 0.15 and 0.30 mg. of uric acid. (Usually a dilution of 1 to 20 is satisfactory.) Ten ml. of the diluted urine are measured into a 50 ml. volumetric flask, 5 ml. of the 5 per cent sodium cyanide solution (*poisonous!*) are added from a buret, followed by 1 ml. of the arsenophosphotungstic acid reagent. The contents of the flask are mixed by gentle shaking, and at the end of 5 minutes diluted to the 50-ml. mark with distilled water and mixed. For colorimetric measurement, this blue solution is then compared with a simultaneously prepared solution obtained by treating 10 ml. of the standard uric acid solution (0.2 mg. of uric acid) in a 50-ml. flask with 5 ml. of the sodium cyanide solution, 1 ml. of the reagent, and diluting to the mark at the end of five minutes. For photometric measurement, determine the densities of unknown and standard at 520 mμ, setting the photometer to zero density with a blank solution obtained by treating 10 ml. of water in a 50-ml. flask with cyanide and reagent exactly as described for unknown and standard.**

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.2 \times \frac{D}{10} = \text{uric acid content of the urine, in g. per liter}$$

D is the dilution (usually 20) of the urine. For most accurate results, the standard and unknown should read within a few mm. of each other. If they differ significantly, repeat the analysis using a greater or lesser dilution of the urine as required.

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[96] Benedict and Franke: *J. Biol. Chem.*, **52**, 387 (1922). For a modification of this method, claimed to be superior, see Christman and Ravwitch: *J. Biol. Chem.*, **95**, 115 (1932). A photometric version of the Christman-Ravwitch procedure is used in the uricase procedure of Buchanan, Block, and Christman described in the text (p. 907).

[97] Solutions Required for Uric Acid Determination: 1. *Reagent.* The reagent employed is the one used in the Benedict procedure for the direct determination of uric acid in blood (Benedict: *J. Biol. Chem.*, **51**, 187 (1922)) and is prepared as follows: 100 g. of pure sodium tungstate (preferably Merck's or J. T. Baker's C. P. product) are placed in a liter pyrex flask and dissolved in about 600 ml. of water. Now add 50 g. of pure arsenic acid (As_2O_5) followed by 25 ml. of 85 per cent phosphoric acid and 20 ml. of concentrated hydrochloric acid. The mixture is boiled for about 20 minutes, cooled, and diluted to 1 liter. The reagent appears to keep indefinitely.

2. *Sodium Cyanide.* A 5 per cent solution of sodium cyanide, containing 2 ml. of concentrated NH_4OH per liter, which should be prepared fresh once in about 6 to 7 weeks.

3. *Uric Acid.* A standard solution of uric acid, acidified with hydrochloric acid, containing 0.2 mg. of uric acid in 10 ml. is employed. This solution may be readily prepared by dilution of Benedict's phosphate standard uric acid solution described in connection with the Newton uric acid method for blood (see p. 563). Measure 50 ml. of the phosphate standard solution (containing 10 mg. of uric acid) into a 500-ml. volumetric flask and dilute to about 400 ml. with distilled water. Add 25 ml. of dilute hydrochloric acid (made by diluting 1 volume of the concentrated acid with 9 volumes of water), and dilute the solution to 500 ml. and mix. This dilute standard solution should be prepared fresh from the phosphate standard every 10 days to 2 weeks.

[98] Uric acid tends to precipitate out of urine on short standing, particularly if the urine is concentrated or acid. Analyses should therefore be carried out as soon as possible after obtaining the urine, and on the well-mixed sample, otherwise results will be misleading.

For photometric measurement:

$$\frac{\text{Density of Standard}}{\text{Density of Unknown}} \times 0.2 \times \frac{D}{10} = \text{uric acid content of the urine, in g. per liter}$$

As above *D*, is the dilution of the urine. The spectrophotometric characteristics of the uric acid color are shown in Fig. 249. At 520 mμ, and in a 1-cm. cuvette, the standard has a density of approximately 0.300. Agreement with Beer's law is satisfactory only at a uric acid content about equal to that of the standard. For higher values, or for photometric measurement at greater depths of solution, carry out the analysis on a greater dilution of the urine. For more accurate results, prepare a calibration curve as described for the following method (p. 908).

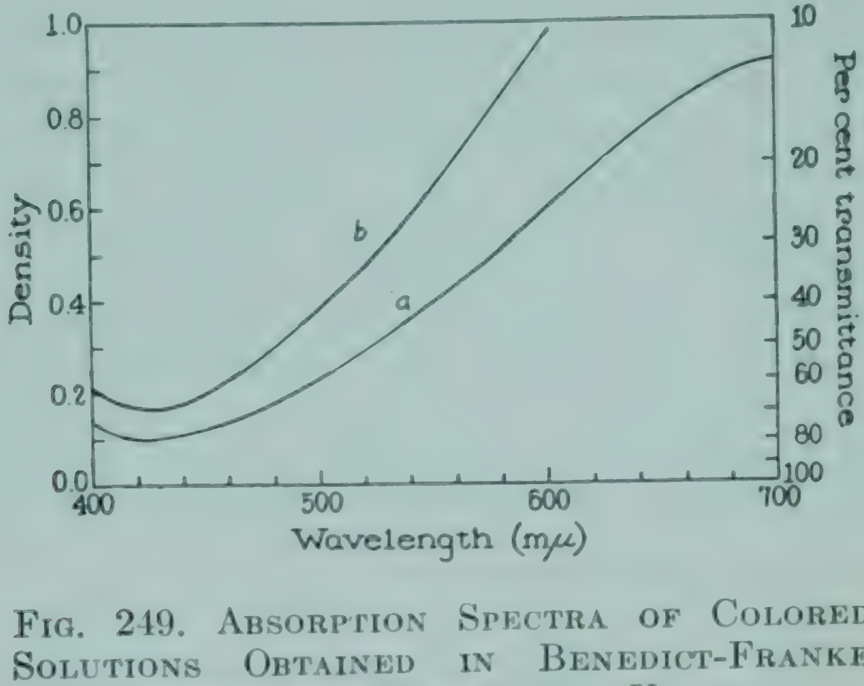

FIG. 249. ABSORPTION SPECTRA OF COLORED SOLUTIONS OBTAINED IN BENEDICT-FRANKE METHOD FOR URIC ACID IN URINE.

For standards containing 0.2 mg. (*a*) and 0.4 mg. (*b*) of uric acid. Solution depth, 1 cm.

Interpretation. For adults on a mixed diet the average excretion of uric acid is about 0.7 g. It arises from the purines of ingested food (exogenous uric acid) and from purines derived from the body tissues by disintegration of nuclear material (endogenous uric acid). This distinction between two metabolic sources of urinary uric acid, first postulated by Folin many years ago, appears to be confirmed by more recent work based on the use of isotopes.[99] Exogenous uric acid depending entirely upon the diet is greatly increased by the ingestion of purine-rich foods (meat, liver, sweetbreads, etc.) and reduced to a very low level on purine-free foods, e.g., milk, eggs, etc. (see table, Chapter 33). Endogenous uric acid is influenced by exercise and by the diet (protein foods particularly giving rise to increases). It appears to be partly the result of gastrointestinal secretory activity. On a purine-free diet the average excretion is 0.1 to 0.5 g. On a high-purine diet the uric acid output may be 2 g. per day.

In gout the uric acid content of the urine is low preceding an attack and increases during the attack, this fall and rise being more or less characteristic. The excretion rises after cincophen administration, apparently because of increased kidney activity, and after the administration of

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[99] Plentl and Schoenheimer: *J. Biol. Chem.*, **153**, 203 (1944).

ACTH. In leukemia the excretion is extremely high due to nuclear destruction. The uric acid content of the urine is of importance in relation to the formation of uric acid calculi. The administration of alkali carbonates and citrates by decreasing the acidity of the urine increases its solvent power for uric acid, and decreases the possibility of the formation of this type of calculus.

2. Uricase Method of Buchanan, Block, and Christman:[100] ***Principle.*** The color intensity resulting from the application of a uric acid color reaction directly to a portion of the diluted urine is determined photometrically. A second portion of urine is incubated with a preparation of the enzyme uricase, which specifically destroys uric acid, and the uric acid color reaction applied to this incubated sample. The difference in color value before and after treatment with uricase is considered to be a measure of the true uric acid content of the urine.

Procedure.[101]

(a) TOTAL COLOR. **Transfer 5 ml. of urine to a 250-ml. volumetric flask, dilute with water to the mark, and mix. Transfer 10 ml. of this diluted urine to a 50-ml. volumetric flask and add 15 ml. of water. In a second similar flask place 25 ml. of water; this serves as a photometric blank. To each flask add 2.5 ml. of urea-cyanide solution, followed by 1 ml. of arsenophosphotungstic**

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[100] Buchanan, Block, and Christman: *J. Biol. Chem.*, **157**, 181 (1945). For a similar procedure see Schaffer: *J. Biol. Chem.*, **153**, 163 (1944).

[101] Reagents Required: *Urea-Cyanide Solution.* Dissolve 25 g. of pure sodium cyanide and 50 g. of anhydrous sodium carbonate in 400 ml, of water. Cool, add 75 g. of pure urea and dilute with water to 500 ml. This solution is usable for several months, even though a slight precipitate will settle out on standing. *It is extremely poisonous and must be handled carefully.*

Arsenophosphotungstic Acid. This is the same reagent that is used in the Benedict-Franke method (see p. 905).

Uricase Powder. Remove the superficial fat from 5 pounds of fresh beef kidneys and grind in a meat-chopper. Transfer to a large wide-mouthed bottle and wash by running in cold tap water slowly through a tube reaching to the bottom of the bottle until the supernatant fluid is quite clear and colorless. Homogenize small portions of the resulting material in a Waring blendor with an approximately equal weight of benzene. To the combined total homogenate add 2 volumes of cold acetone. Allow the precipitate to settle, filter through cheesecloth or a towel, and squeeze dry. Suspend the solid material in about three times its weight of acetone, allow it to settle, and again filter off. Repeat until the resulting powder is thoroughly dehydrated and defatted. Spread the material on towels and allow to dry in air overnight. Screen the powder through a 40-mesh sieve and store in a vacuum desiccator.

This powder should give no blank color when carried through the incubation procedure and subsequent color reaction described in the text. To test for *activity*, prepare a special lithium carbonate solution of uric acid containing 1 g. of uric acid and 0.6 g. of lithium carbonate per liter by following the procedure described on p. 560 for the preparation of the Folin stock uric acid solution *up to* but *not including* the addition of formaldehyde and sulfuric acid, which if present will interfere with uricase activity. Each ml. of this solution contains 1 mg. of uric acid. Incubate 3 to 4 ml. of this solution with 250 mg. of the uricase powder, borate buffer, etc., and continue with the color reaction as for the analysis of an unknown. If the uricase is active, no residual color will be obtained. The special lithium carbonate standard is unstable and must be made up fresh on the day of use.

Standard Uric Acid Solution. The Folin stock standard solution is used, containing 1 mg. of uric acid per ml., prepared as described on p. 560 in connection with the determination of uric acid in blood. Suitable dilutions of this stock standard are used in the preparation of the calibration curve, as described in the text. Dilute standards are unstable and must be prepared fresh on the day of use.

Borate Buffer (pH 9.2). Dissolve 12.4 g. of boric acid in 1 liter of 0.1 N sodium hydroxide solution.

10 Per Cent Sodium Tungstate, 0.66 N Sulfuric Acid. The same reagents used in the preparation of Folin-Wu blood filtrates (see p. 543).

acid. Both of these reagents are extremely poisonous; they should be handled with care, and always dispensed from burets. Immediately after the addition of the arsenophosphotungstic acid, dilute the contents of the flask to the 50-ml. mark with water and mix thoroughly. Allow to stand for exactly 30 minutes after so diluting (in serial analyses a suitable time schedule should be established), then determine the density of the unknown in a photometer at 690 mμ, setting the photometer to zero density with the blank.

(b) RESIDUAL COLOR. **Place 5 ml. of the original undiluted urine in a small flask, add 10 ml. of water and a few drops of 0.04 per cent thymol blue solution as indicator. Titrate with 0.1 N sodium hydroxide solution to a definite blue tint. Record the amount of alkali required, and discard the mixture. Transfer a fresh 5-ml. portion of the urine to a 50-ml. volumetric flask, and add the predetermined amount of alkali (without indicator). Add 250 mg. of uricase powder, 5 ml. of borate buffer (pH 9.2), and wash down the sides of the flask with 10 ml. of water. Place in a water bath at 45° C. for 2 hours. Add 1 ml. of 10 per cent sodium tungstate and 1.5 ml. of two-thirds normal sulfuric acid. Dilute to 50 ml. with water, mix well, and pour onto a dry filter. Transfer 10 ml. of the filtrate to a 50-ml. volumetric flask, add 15 ml. of water, and continue with the addition of the color reagents and measurement exactly as described above for "Total Color" procedure. The same reagent blank may be used for setting the photometer to zero density, or a 5-ml. portion of water may be treated with uricase, buffer, etc., as described for the analysis of the urine, and the final solution after treatment with the color reagents used as a photometric blank.**

CALCULATION.

a. Total Color Value. Determine the amount of uric acid in mg. equivalent to the photometer reading of the total color sample by reference to a calibration curve (see below). Multiply this by 5 (since the 10 ml. of urine diluted 1:50 represents 0.2 ml. of original urine) to obtain the uric acid content for the undiluted urine equivalent to the total color, in mg. per ml. (or g. per liter).

b. Residual Color Value. In a similar way, determine the uric acid content equivalent to the residual color; the value in mg. obtained from the calibration curve gives directly the residual color equivalent of the original urine, in mg. per ml., or g. per liter, since 10 ml. of urine diluted 1:10 are used.

c. True Uric Acid Content. Subtract the "residual color" value from the "total color" value, to obtain the true uric acid content of the urine:

$$\begin{array}{ccccc} \text{Total Color} & - & \text{Residual Color} & = & \text{True uric acid content} \\ \text{(as uric acid)} & & \text{(as uric acid)} & & \text{(in g. per liter)} \end{array}$$

Multiply the result by the urine volume (expressed in liters) to obtain the uric acid content of the entire sample.

Other dilutions and aliquots than those specified may be used if the final colored solutions are either too light or too dark for accurate photometric measurement; in such case the calculations must be corrected accordingly.

The use of a calibration curve prepared from standard uric acid solutions is recommended because of the deviation from Beer's law shown by lower concentrations of uric acid. To prepare such a curve, proceed as follows: Dilute 1 ml. of the stock Folin uric acid standard (containing 1 mg. per ml.) to 100 ml. with water and mix, thus obtaining a solution containing 0.01 mg. of uric acid per ml. Measure aliquots of this dilute standard into 50-ml. volumetric flasks (preferably in duplicate) to give a series of solutions of known uric acid content covering the range of satisfactory photometric measurement (0.01 to 0.12 mg. for measurement at 2 cm. solution depth or its approximate equivalent, as with the Evelyn photometer; 0.02 to 0.24 mg. if 1-cm. cuvettes or their equivalent are to be used). Include a blank flask containing water alone in the series. Adjust the volume in each flask to 25 ml. by adding water where necessary, then add 2.5 ml. of urea-cyanide solution to each flask. At timed (e.g., one-minute) intervals add 1 ml. of arsenophosphotungstic acid to a flask, dilute immediately to 50 ml. with water, and mix thoroughly. At the end of 30 minutes, set the photometer to zero density with the blank, and determine the density for each standard exactly 30 minutes after diluting and mixing. Plot the determined densities against the amount of uric acid present (in mg.) on cross-section paper, and draw a smooth curve to include the points.

In an analysis, the determined density of an unknown is translated into its equivalent uric acid value by reference to the curve. For accurate results the curve should be checked at intervals, particularly if new reagents are made up, and reconstructed if necessary. In an analysis, environmental and other conditions should reproduce as consistently as possible those prevailing at the time the curve was established. For further discussion concerning the validity and use of calibration curves in photometric analysis, see the section "Photometry" in Chapter 23, p. 512.

Interpretation. Results by the uricase method described here indicate that only 80 to 90 per cent of the color obtained by direct treatment of urine with uric acid color reagents may actually be due to uric acid; this discrepancy may be much greater if the diet contains significant amounts of the methyl xanthines (caffeine, theophylline, and theobromine, found in coffee, tea, and cocoa). In such instances, the uricase method appears to be even more specific for uric acid than the "isolation" procedures (see following method). For further aspects of interpretation, see under previous method.

3. Colorimetric Methods of Folin:[102] ***Principle.*** Phosphotungstic acid is reduced by uric acid with the production of a blue color which is compared with that produced with a standard solution of uric acid. Polyphenols also react, thus giving too high results, and amino acids decrease the color. The direct application of the reaction to urine is not therefore an accurate method but may be useful for many clinical purposes. The indirect method in which the uric acid is first separated by precipitation with silver nitrate is claimed to be an accurate method if conditions are strictly followed.

Procedure.

(a) INDIRECT METHOD. **Half fill a 100-ml. volumetric flask with water. With a Folin-Ostwald pipet introduce 1 ml. of the urine. Add 10 ml. of the chloride-acetate solution[103] and then without shaking so as to avoid foaming, dilute to the mark with water and mix.**

Transfer 5 ml. of the diluted urine to one 15-ml. centrifuge tube and 3 ml. plus 2 ml. of water to another. To each add 3 ml. of the silver nitrate solution

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[102] Folin: *J. Biol. Chem.*, **101**, 111 (1933); **106**, 311 (1934).

[103] *Chloride-Acetate Solution.* A solution containing 1 per cent NaCl, 2 per cent cryst. sodium acetate, and 1 volume per cent of 99 per cent acetic acid.

5 Per Cent Solution of Silver Nitrate. This solution, even if perfectly clear when first prepared, may develop a slight color on standing. This color is most quickly produced by heating to 100° C. for 2 hours in a flask covered with a beaker. After cooling, add a few ml. of a solution containing 50 mg. of NaCl, shake thoroughly and filter through a double layer of quantitative filter paper until crystal clear. Thereafter the solution will remain perfectly colorless and need not be kept in brown bottles.

Other solutions are the same as those used in blood methods (see p. 560).

and centrifuge at once fairly rapidly for 4 to 5 minutes so as to get perfectly clear supernatant solutions. A few tiny flakes may float on the surface but these contain no uric acid. Decant and drain over a sink. It is permissible to let cold tap water rinse the mouths of the tubes during the draining. With a 25-ml. cylinder or a buret, to each tube add 10 ml. of the urea-cyanide solution (*poisonous!*). Stir immediately and simultaneously with glass rods until the two sediments have completely dissolved. Transfer the silver cyanide solutions to test tubes graduated at 25 ml. and rinse with exactly 5 ml. of water. Mix by whirling at an angle of about 60° until the solutions are visibly uniform. In another graduated test tube place 5 ml. of the standard uric acid solution containing 0.02 mg. of uric acid together with 10 ml. of the urea-cyanide solution and mix.

With a 10-ml. blood pipet add to each of the three tubes 4 ml. of the uric acid reagent and let stand for 20 minutes. Dilute to volume and mix. For colorimetric measurement, make the comparison between the standard and the unknown which is nearest to it in depth of color. When the standard is set at 20 mm., colorimetric readings between 35 mm. and 10 mm. are acceptable. For photometric measurement, only one unknown is necessary. Determine its density, and that of the standard, in a photometer at 660 mμ, setting the photometer to zero density with a blank prepared by treating 5 ml. of water with urea-cyanide and color reagent, etc., exactly as described for the preparation of the standard.

(b) DIRECT METHOD. Half fill a 100-ml. volumetric flask with water. With a Folin-Ostwald pipet introduce 1 ml. of urine, dilute to volume and mix. Introduce into test tubes graduated at 25 ml., 5 ml. of the diluted urine and 3 ml. of the diluted urine plus 2 ml. of water. To another graduated test tube add 5 ml. of the standard uric acid solution. Add 10 ml. of the urea-cyanide solution (*poisonous!*) to each, mix, and add 4 ml. of the uric acid reagent. Let stand for 20 minutes. Dilute to volume and mix. Continue with colorimetric or photometric measurement as described above for the indirect method.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.02 \times \frac{100}{v} = \begin{array}{c}\text{Uric acid content of undiluted}\\ \text{urine, in g. per liter}\end{array}$$

v is the volume of *diluted* urine (5 ml. or 3 ml.) used in an analysis. The same calculations are used for both indirect and direct methods.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.02 \times \frac{100}{v} = \begin{array}{c}\text{Uric acid content of undiluted}\\ \text{urine, in g. per liter}\end{array}$$

The significance of v is the same as for colorimetric measurement. The conditions for satisfactory photometric measurement are the same as those given on p. 561 in connection with the determination of uric acid in blood, since the same standards and color reaction are used.

Interpretation. See previous methods.

4. Folin-Shaffer Method: Principle. Phosphates and some organic matter are removed by means of uranium acetate. The uric acid is precipitated as ammonium urate which is titrated with potassium permanganate.[104]

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[104] Folin and Shaffer: *Z. physiol. Chem.*, 32, 552 (1901). This method was described in the 11th and previous editions of this book.

PURINE BASES (INCLUDING URIC ACID)

1. Krüger and Schmid's Method: Principle. This method serves for the determination of both uric acid and the purine bases. The principle involved is the precipitation of both the uric acid and the purine bases in combination with copper oxide and the subsequent decomposition of this precipitate by means of sodium sulfide. The uric acid is then precipitated by means of hydrochloric acid and the purine bases are separated from the filtrate in the form of their copper or silver compounds. The nitrogen content of the precipitates of uric acid and purine bases is then determined by means of the Kjeldahl method (see p. 874) and the corresponding values for uric acid and purine bases calculated. For a study of this method as applied to the determination of purines in protein-free tissue extracts, on a microscale, see Hitchings and Fiske.[105]

Procedure. **To 400 ml. of albumin-free urine[106] in a liter flask,[107] add 24 g. of sodium acetate, and 40 ml. of a solution of sodium bisulfite,[108] and heat the mixture to boiling. Add 40 to 80 ml.[109] of a 10 per cent solution of copper sulfate and maintain the temperature of the mixture at the boiling point for at least 3 minutes. Filter off the flocculent precipitate, wash it with hot water until the wash water is colorless, and return the washed precipitate to the flask by puncturing the tip of the filter paper and washing the precipitate through by means of hot water. Add water until the volume in the flask is approximately 200 ml., heat the mixture to boiling, and decompose the precipitate of copper oxide by the addition of 30 ml. of sodium sulfide solution.[110] After decomposition is complete, the mixture should be acidified with acetic acid and heated to boiling until the separating sulfur collects in a mass. Filter the hot fluid with the aid of a filter pump, wash with hot water, add 10 ml. of 10 per cent hydrochloric acid, and evaporate the filtrate in a porcelain dish until the total volume has been reduced to about 10 ml. Permit this residue to stand about 2 hours to allow for the separation of the uric acid, leaving the purine bases in solution. Filter off the precipitate of uric acid, using a small filter paper, and wash the uric acid, with water made acid with sulfuric acid, until the total volume of the original filtrate and the wash water aggregates 75 ml. Determine the nitrogen content of the precipitate by means of the Kjeldahl method (see p. 874), and calculate the uric acid equivalent.[111]**

Render the filtrate from the uric acid crystals alkaline with sodium hydroxide, add acetic acid until faintly acid, and heat to 70° C. Now add 1 ml. of a 10 per cent solution of acetic acid and 10 ml. of a suspension of manganese dioxide[112] to oxidize the traces of uric acid which remain in the solution.

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[105] Hitchings and Fiske: *J. Biol. Chem.*, **140**, 491 (1941).

[106] If albumin is present, the urine should be heated to boiling, acidified with acetic acid, and filtered.

[107] The total volume of urine for the 24 hours should be sufficiently diluted with water to make the total volume of the solution 1600 to 2000 ml.

[108] A solution containing 50 g. sodium bisulfite in 100 ml. water.

[109] The exact amount depending upon the content of the purine bases.

[110] This is made by saturating a 1 per cent solution of sodium hydroxide with hydrogen sulfide gas and adding an equal volume of 1 per cent sodium hydroxide. Ordinarily the addition of 30 ml. of this solution is sufficient, but the presence of an excess of sulfide should be *proved* by adding a drop of lead acetate to a drop of the solution. Under these conditions a dark brown color will show the presence of an excess of sodium sulfide.

[111] This may be done by multiplying the nitrogen value by three and adding 3.5 mg. to the product as a correction for the uric acid remaining in solution in the 75 ml.

[112] Made by heating a 0.5 per cent solution of potassium permanganate with a little alcohol until it is decolorized.

Agitate the mixture for 1 minute, add 10 ml. of the sodium bisulfite solution[113] and 5 ml. of a 10 per cent solution of copper sulfate, and heat the mixture to boiling for 3 minutes. Filter off the precipitate, wash it with hot water, and determine its nitrogen content by means of the Kjeldahl method (see p. 874). Inasmuch as the composition and proportion of the purine bases present in urine is variable, no factor can be applied. The result as regards these bases must therefore be expressed in terms of nitrogen.

Benedict and Saiki report cases in which the total purine nitrogen by this method was less than the uric acid nitrogen as determined by the Folin-Shaffer method. The inaccuracy was found to lie in the Krüger and Schmid method. To obviate this they advise the addition of 20 ml. of glacial acetic acid for each 300 ml. of urine employed, the acid being added before the first precipitation.

Interpretation. The amount of purine bases excreted by a normal man is small and variable. Values from 16 to 60 mg. have been found. The purine base nitrogen is of course only a fraction of this. The amount excreted is influenced by the diet somewhat in the same way as is the excretion of uric acid, being also increased in disorders associated with increased uric acid excretion such as leukemia. The purine bases form a higher percentage of the total purine excretion in the case of the monkey, sheep, and goat than in the case of man.

2. Hunter and Givens' Modification of the Krüger-Schmid Method:[114] ***Principle.*** The Krüger-Schmid process is combined with the colorimetric method for uric acid (see p. 909).

Procedure. **The first copper-purine precipitate as obtained in the Krüger-Schmid procedure is suspended in about 200 ml. of water, to which there is added about 1 ml. of concentrated hydrochloric acid. The mixture is vigorously boiled, whereupon the whole or greater part of the precipitate goes into solution. Removal of the copper is effected by treatment, while hot, with hydrogen sulfide and excess of the sulfide is completely expelled by renewed boiling. Filtration under suction, and thorough washing of flask and filter result in a filtrate which is perfectly clear and nearly colorless. This is concentrated if necessary, and made up to a convenient volume which must of course be sufficiently large to retain, when cool, the uric acid in solution. Of this an aliquot part is utilized directly for the colorimetric determination of uric acid. In the remainder the residual uric acid is destroyed and bases determined according to the regular Krüger-Schmid procedure. This modification is recommended particularly where the amount of uric acid present is minute.**

3. Welker's Modification of the Methods of Arnstein and of Salkowski: ***Principle.*** The phosphates are removed by treatment with magnesia mixture. The purine bases and uric acid are then thrown down as their silver salts and the nitrogen content of this precipitate determined.[115]

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[113] To dissolve the excess of manganese dioxide.

[114] Hunter and Givens: *J. Biol. Chem.*, **17**, 37 (1914).

[115] Dittman and Welker: *N. Y. Med. J.*, **89**, 1134 (1909).

ALLANTOIN

1. Method of Larson:[116] ***Principle.*** The urine is treated with phosphotungstic acid to remove interfering substances. Basic lead acetate is then added to remove excess phosphotungstic acid and residual interfering substances. Excess lead is removed by sulfuric acid and excess acid neutralized by sodium hydroxide. The solution is then boiled with Folin ammoniacal reagent and acid molybdate reagent added to the cooled solution. After proper dilution a colorimetric comparison is made against a 1-mg. allantoin standard.

Procedure. **Transfer 1.5 g. of phosphotungstic acid[117] to a 50-ml. centrifuge tube and add 5 ml. of water. Rotate gently to insure solution; then add 5 ml. of animal urine. Centrifuge immediately, place the tube in a refrigerator for one-half hour, then centrifuge again until perfectly clear. The addition of a crystal of phosphotungstic acid should not cause further precipitation. Add 5 ml. of basic lead acetate solution which precipitates the excess phosphotungstic acid as well as the remaining interfering substances. Centrifuge the mixture, then treat with 5 ml. of 5 per cent sulfuric acid to remove the excess lead, and centrifuge until perfectly clear. Pipet 2 ml. of the resulting water-clear liquid into a Folin-Wu sugar tube, neutralize with 5 per cent sodium hydroxide, and then add 2 ml. of Folin ammoniacal copper reagent.[118] Place the tubes in a rapidly boiling water bath for 10 minutes, cool, then add 2 ml. of acid molybdate reagent.[119] Dilute the tubes to volume and read in a**

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[116] Larson: *J. Biol. Chem.*, **94**, 727 (1932). According to Young and Conway (*J. Biol. Chem.*, **142**, 839 (1942)), the Larson procedure, although giving reasonably good recovery of added allantoin, gives somewhat high results on urine compared to the procedure they describe, which is claimed to be more specific and satisfactory. For details, see the original article. For a photometric version of the Young-Conway procedure, and method for the determination of allantoin in blood, see Young, MacPherson, Wentworth, and Hawkins: *J. Biol. Chem.*, **152**, 245 (1944).

[117] *Phospho-24-tungstic Acid.* Dissolve 100 g. of $Na_2WO_4 \cdot 2H_2O$ in about 100 ml. of water with the aid of heat. Add 10 ml. of 85 per cent H_3PO_4 and then 80 ml. of concentrated HCl. Cool. After 4 hours or more, filter on a Buchner funnel and suck as dry as possible. Redissolve the precipitate in 120 ml. of H_2O, pour the solution into a liter separatory funnel, add about 90 ml. of ether, and then add 40 ml. of concentrated HCl. Shake. After standing a few minutes, there should be three layers of liquid. The lowest layer contains nearly all the complex acid. If there are only two layers, more ether must be added and the mixture shaken again. Transfer the lowest layer to another separatory funnel, add about 120 ml. of water, and shake vigorously; then add 50 ml. of ether and finally 50 ml. of concentrated HCl. After standing, the lowest layer, which should be perfectly clear, is transferred to a crystallizing dish. Add 30 ml. of H_2O and 1 drop of liquid bromine and evaporate on a steam bath. The solution should be greenish in color. If the slightest trace of dust or organic matter is present, a pinkish color develops; and 1 or 2 drops more of liquid bromine must be added to oxidize this foreign material.

Evaporate on the steam bath until crystals begin to form on the surface. Let stand overnight. The crystals obtained are sucked as dry as possible on a large Buchner funnel. After air-drying for one week, powder the crystals and keep in an amber glass container. This phosphotungstic acid should dissolve instantly to give a perfectly clear, practically colorless solution.

[118] *Folin Ammoniacal Copper Solution.* Dissolve 100 g. of ammonium sulfate in about 400 ml. of water and filter into a volumetric liter flask. 100 ml. of 10 per cent sodium hydroxide are then added, 12 g. of sodium tartrate, and finally a solution of 5 g. of copper sulfate. Dilute to volume and mix. This reagent will not give a blank for months, if kept in the dark in well-filled, tightly stoppered, amber glass bottles. The bottles should be of small volume.

[119] *Folin Acid Molybdate Reagent.* Prepare a stock solution of 30 per cent brominated sodium molybdate as follows: Dissolve 300 g. of sodium molybdate in water and make up to 1 liter. The solution is slightly turbid. Add 2 or 3 drops of liquid bromine and let stand

colorimeter against a 1-mg. allantoin standard.[120] Photometric data on this procedure are not available.

CALCULATION.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 1 \times \frac{100}{5} = \text{mg. allantoin per 100 ml.}$$

Interpretation. Allantoin is found in only small amounts in human urine (35 to 45 mg. per day), and appears to be mainly, though not entirely, exogenous in origin. It forms, however, the principal end product of the purine metabolism of practically all mammals other than man and the anthropoid apes, with the notable exception of the pure-bred Dalmatian coach hound which excretes a considerable fraction of its purine nitrogen as uric acid. Thus over 90 per cent of the purine-allantoin nitrogen excretion of the dog, the cow, and the pig occurs as allantoin. In these animals its origin is from exogenous and endogenous purines, and its excretion is influenced by much the same factors as is that of uric acid in man. It appears to be entirely a waste product, since if injected into the blood of man or dogs it is excreted almost quantitatively in the urine.[121]

HIPPURIC ACID

1. Method of Griffith:*[122] *Principle. Hippuric acid is extracted from urine with ether in a continuous extraction apparatus. The residue obtained by distilling off the ether is treated with bromine and sodium hypobromite to destroy traces of urea. Hippuric acid nitrogen is then determined in the residue by the Kjeldahl procedure.

In Quick's method[123] the hippuric acid is extracted in a similar manner, and the amino nitrogen determined by the formol titration (see p. 897). The older methods involved hydrolysis of the hippuric acid and titration of the liberated benzoic acid. These may give misleading results, however, because benzoic acid may be present also in the form of glucuronic acid monobenzoate (benzoyl glucuronic acid).

Procedure. **Place 10 ml. of protein-free urine (containing not more than 150 mg. of hippuric acid) and 0.1 ml. of concentrated HCl in the extraction tube and 100 ml. of ether in the 500-ml. Kjeldahl flask. (The arrangement of the extraction apparatus is shown in Fig. 250.) Immerse the lower portion of the flask in a water bath heated to 60° to 70° C. and continue the extraction for one hour. Distil off the ether. To the dry residue in the flask add 5 ml. of sodium hypobromite[124] solution and shake one minute. Add 2 ml. of sulfuric acid (diluted 1:5). Mix thoroughly. Add 2 ml. of 25 per cent NaOH and 2 ml. of the hypobromite solution and shake 1 minute. Proceed with the determination of nitrogen in the flask by the Kjeldahl method.**

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overnight. Transfer 500 ml. of the clear supernatant liquid to a liter flask and add, with stirring, 225 ml. of 85 per cent phosphoric acid. Then add 150 ml. of cool sulfuric acid (25 volumes per cent). The bromine which is liberated is removed by aeration. Add 75 ml. of 99 per cent acetic acid, mix, and dilute to 1 liter.

[120] *Allantoin Standard.* Dissolve 100 mg. of allantoin in about 50 ml. of water with the aid of heat, but do not allow to boil. Cool, transfer to a 100-ml. volumetric flask, and dilute to volume. Cover with toluene. Such a standard will keep for about 2 weeks at room temperature without deterioration. Without toluene, a loss of 1 per cent of the allantoin is noted after standing 1 week. Givens reports a loss of 1.7 per cent after 90 days.

[121] Young, Wentworth, and Hawkins: *J. Pharmacol.*, **81**, 1 (1944).

[122] Griffith: *J. Biol. Chem.*, **69**, 197 (1926). Also private communication.

[123] Quick: *J. Biol. Chem.*, **67**, 477 (1926).

[124] Prepared by mixing equal volumes of 25 per cent NaOH and a solution containing 12.5 g. of bromine and 12.5 g. of sodium bromide dissolved in 100 ml. of water.

CALCULATION. If y represents the number of ml. of 0.1 N acid neutralized by ammonia in the Kjeldahl titration, then

$$y \times 1.4 \times \frac{179}{14} = \text{mg. hippuric acid in 10 ml. urine}$$

Interpretation. The average excretion of hippuric acid by a normal adult man is about 0.7 g. per day. The amount is increased by the ingestion of benzoic acid or fruits such as plums, prunes, and cranberries which contain, in addition to benzoic acid, certain other precursors of hippuric acid (quinic acid, etc.). It arises in part apparently from putrefaction products formed in the intestine. In herbivora it is often the most abundant nitrogenous constituent of the urine.

2. Hippuric Acid Test for Liver Function (***Quick***[125]): ***Principle.*** If benzoic acid is ingested or injected, a major portion of it combines with glycine to form hippuric acid which is then excreted in the urine (for reactions, see p. 440). In man (but not necessarily in other animals) this synthesis appears to take place primarily in the liver. If the liver is damaged, the amount of hippuric acid excreted is diminished relative to that found normally. It is claimed that clinically the decrease in hippuric acid excretion under test conditions is fairly proportional to the extent of hepatic impairment.

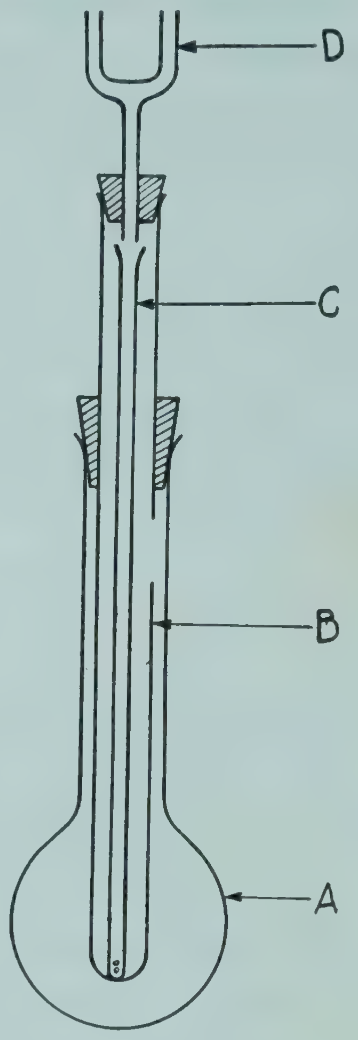

FIG. 250. CONTINUOUS EXTRACTION APPARATUS (GRIFFITH).

A, 500-ml. Kjeldahl flask; *B*, glass tube (420 × 15 mm.) with side opening; *C*, glass tube (400 × 6 mm.) with widened top and small openings at bottom; *D*, condenser.

Procedure. **Just before the test, which is preferably given in the morning about 1 hour after a light breakfast (cereals or toast, and coffee, tea, or milk), the patient is instructed to empty his bladder as completely as possible, and the urine obtained is discarded. A dose of 6 g. of sodium benzoate dissolved in 30 ml. of water (flavored with oil of peppermint if desired) is then ingested,[126] followed by half a glass of water. More water may be taken during the test if necessary but excessive water intake should be discouraged in order to keep the urine volume down. A complete specimen of urine is collected each hour after the test dose, for 4 hours.**

Measure the volume of each hourly specimen in a graduate. If a specimen volume exceeds 150 ml., it is advisable to acidify slightly with acetic acid and to concentrate the sample on a water bath to about 50 ml. Combine the four specimens in a graduated cylinder, measure the volume, and transfer to a beaker. Add solid ammonium sulfate in the proportion of 5 g. for each 10 ml. of urine, and stir to dissolve. Filter or centrifuge. To the clear filtrate or centrifugate in a beaker add sufficient concentrated hydrochloric acid (usually about 1 ml. is required) to render distinctly acid to Congo red or thymol blue (pH about 2).[127] Stir vigorously with a glass rod, scratching the sides of

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[125] Quick: *Am. J. Clin. Path.*, **10**, 222 (1940). See also Weichselbaum and Probstein: *J. Lab. Clin. Med.*, **24**, 636 (1938–1939): Hepler and Gurley: *ibid.*, **27**, 1593 (1941–1942).

[126] For details of the *intravenous* test, see Quick (*loc. cit.*).

[127] Indicator paper may also be used.

the beaker to promote crystallization, then place in the refrigerator or in ice water for 30 minutes. Filter off the crystalline hippuric acid by suction on a small Buchner funnel, and wash the precipitate with several small portions of ice-cold distilled water, using the wash water to complete the transfer of the crystals from the beaker to the Buchner funnel.

Quantitatively transfer the precipitate and filter paper to a beaker (a little water may be used to aid in the transfer), add sufficient water to cover, and heat to dissolve. Add a few drops of phenolphthalein solution and titrate with 0.5 N sodium hydroxide solution to a permanent pink. Record the buret reading.

CALCULATION. Each ml. of 0.5 N sodium hydroxide is equivalent to 0.0895 g. hippuric acid. Therefore:

$$\text{ml. } 0.5\text{ N alkali used} \times 0.0895 = \text{g. hippuric acid titrated}$$

To determine the amount of hippuric acid excreted, a correction for the solubility of the compound must be added. In the presence of ammonium sulfate as described, the solubility has been established as 0.001 g. per ml. of urine. If V is the total volume of urine in milliliters before adding the ammonium sulfate, the solubility correction is $V \times 0.001$ g. Therefore:

$$(\text{ml. of } 0.5\text{ N alkali used} \times 0.0895) + (V \times 0.001) = \text{g. hippuric acid excreted}$$

$$\text{G. hippuric acid} \times 0.68 = \text{g. benzoic acid}$$

Interpretation. Under the conditions of this test, the average healthy adult will excrete 3.0 to 3.5 g. (or even more) of benzoic acid in the form of hippuric acid. There is some indication that increased excretion above 3 g. is roughly proportional to body weight (or surface area) in normal individuals.[128] Any excretion of 2.7 g. or more is considered nonpathological. Marked diminution in output is found in various liver disorders. For further aspects of clinical interpretation, see texts on clinical diagnosis.

LACTIC ACID

Method of Friedemann and Graeser:[129] ***Principle.*** By treatment with phosphoric acid and potassium permanganate, the lactic acid is converted to acetaldehyde.

$$CH_3{\cdot}CHOH{\cdot}COOH \rightarrow CH_3CHO + CO + H_2O$$

The aldehyde is bound with sodium bisulfite.

$$CH_3CHO + NaHSO_3 \rightarrow CH_3CHOH{\cdot}SO_3Na$$

The bound sulfite is titrated iodimetrically.

Procedure. **A 10- to 25-ml. sample of urine is introduced into a 250-ml. volumetric flask, 10 ml. of 20 per cent $CuSO_4{\cdot}5H_2O$ and 10 ml. of a suspension of $Ca(OH)_2$ are added.[130] The suspension is diluted to the mark and filtered, an aliquot representing not more than 5 ml. of urine being used for analysis.**

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[128] Hepler and Gurley: *loc. cit.*

[129] Friedemann and Graeser: *J. Biol. Chem.*, **100**, 291 (1933). The authors describe adaptations of this method for blood (see p. 624), milk, culture media, and tissues.

[130] Special Reagents: *Calcium Hydroxide Suspension.* 1 kg. of fresh unslaked lime is slaked with water and immediately afterward sufficient water is added to bring the volume

Ten ml. of H_3PO_4-$MnSO_4$ reagent and a pinch of finely powdered talc are placed in a 300-ml. Kjeldahl flask, followed by the sample of urine. About 85 ml. of water are added and the flask attached to the apparatus (see Fig. 251). Ten ml. of $NaHSO_3$ solution are placed in the 150-ml. extraction flask (receiver). The microburner is adjusted to bring the solution to boiling in about 3 minutes. Addition of oxidizing agent is begun as soon as vapors appear in the condenser. The rate of addition is unimportant, but it is essential to have an excess of oxidizing agent throughout the distillation, as indicated by a brownish (not gray) color. It is best to add from 25 to 40 ml. over a 15-minute oxidation period. Shortly before the end of the oxidation the receiving flask is detached from the stopper and lowered. The glass tip is rinsed and the flask containing a total volume of 50 to 75 ml. is cooled prior to titration.

For the removal of excess bisulfite 1 ml. of starch solution is added, followed by a slight excess of the strong iodine solution which is immediately removed by the cautious addition of 0.1 N thiosulfate. The walls of the flask are now washed down by a thin stream of water after which the end point is adjusted to a faint blue with dilute iodine solution.

The flask is cooled and approximately 15 ml. of saturated $NaHCO_3$ are added. The solution is titrated with dilute standard iodine solution which is run in rapidly so as to keep pace with the decomposition into aldehyde and

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to approximately 5 liters. The suspension is thoroughly shaken, allowed to stand for a few seconds, and decanted from the coarser particles.

Phosphoric Acid-Manganous Sulfate Reagent. 100 g. of $MnSO_4.4H_2O$ are dissolved in about 500 ml. of warm water. To this are added 50 ml. of syrupy phosphoric acid (85 per cent, 15 M). The solution is cooled and diluted to a volume of approximately 1 liter.

Oxidizing Agent. To 10 ml. of 0.1 N $KMnO_4$, diluted to approximately 800 ml., are added 2 ml. of the H_3PO_4-$MnSO_4$ reagent, stirring while adding. The volume is brought to about 1000 ml. and the solution is again well mixed. The reduction is complete in 5 to 10 minutes.

Sodium Bisulfite. 25 g. are dissolved in 2 liters of water. The solution should be kept in a stoppered bottle.

Starch Indicator. 5 g. of arrowroot starch are suspended in 10 to 20 ml. of cold water and poured into 500 ml. of boiling water; 500 ml. of hot water are added and boiling is continued for 15 minutes. The flask is covered with a beaker, cooled, and kept in the refrigerator. The supernatant clear solution only is used. The solution will keep several weeks if care is taken to avoid bacterial growth.

Strong Iodine Solution. 40 g. of iodine and 75 g. of KI are dissolved in a small quantity of water and the volume is brought up to about 2 liters.

Standard Iodine Solution. The weak iodine solutions (0.01 to 0.002 N) may be prepared either by dilution of a standard iodine solution or by liberation of iodine from 0.1 N iodate + KI. The latter method is preferred because of the permanence of iodate solutions. 0.1 N $KH(IO_3)_2$ solution contains 3.2496 g. per liter. 0.1 N KIO_3 contains 3.567 g. per liter. Five to 10 g. of c.p. KI, about 200 ml. of cool, distilled water, and 2 to 5 ml. of 5 N H_2SO_4 are placed in a volumetric flask. Standard iodate is added and the volume is brought almost to the mark with cooled water. It is then warmed to 20° C. and the volume adjusted to the mark. Since iodine is volatile even from dilute solutions, they should be kept cool.

Lactic Acid Standard. Lithium lactate is preferred because this salt is anhydrous and not hygroscopic. It is prepared as follows:

U.S.P. lactic acid (85 per cent) is diluted with an equal volume of water and a few drops of phenol red indicator are added. Saturated (approximately 20 per cent) lithium hydroxide or $LiCO_3$ (the former is preferred) solution is added to slight excess, as is indicated by the phenol red. The solution is heated to boiling and the alkali is again added to slight alkalinity. It is now cooled. Four volumes of 95 per cent alcohol are added and after cooling for some time, the mass of crystals is filtered off on a Buchner funnel and washed thoroughly with 95 per cent alcohol. This preparation is recrystallized from water and dried at 100° C.

9.60 g. of lithium lactate are transferred to a 1-liter volumetric flask. Enough H_2SO_4 is added to bring the final concentration to 0.2 N when diluted to the mark. This 0.1 M lactic acid standard will keep for at least one year, provided it is kept away from strong light or stored in a refrigerator.

bisulfite. When this slows up, 1 ml. of 10 per cent Na_2CO_3 is added until the end point persists for at least ½ minute.

Blank determinations should be run with filtrates prepared with the precipitating reagent and the $CuSO_4$ and lime reagents.

CALCULATION. Each ml. of 0.01 N iodine solution used in the titration of bound sulfite is equivalent to 0.45 mg. of lactic acid.

Interpretation. The lactic acid concentration is variable, depending upon the volume, from 4 mg. in very dilute urine to as high as 25 mg. per 100 ml. in concentrated urine. These values are increased after severe exercise and probably in such pathological conditions as are accompanied by deficient oxidative metabolism. For a discussion of lactic acid in the urine, see p. 812.

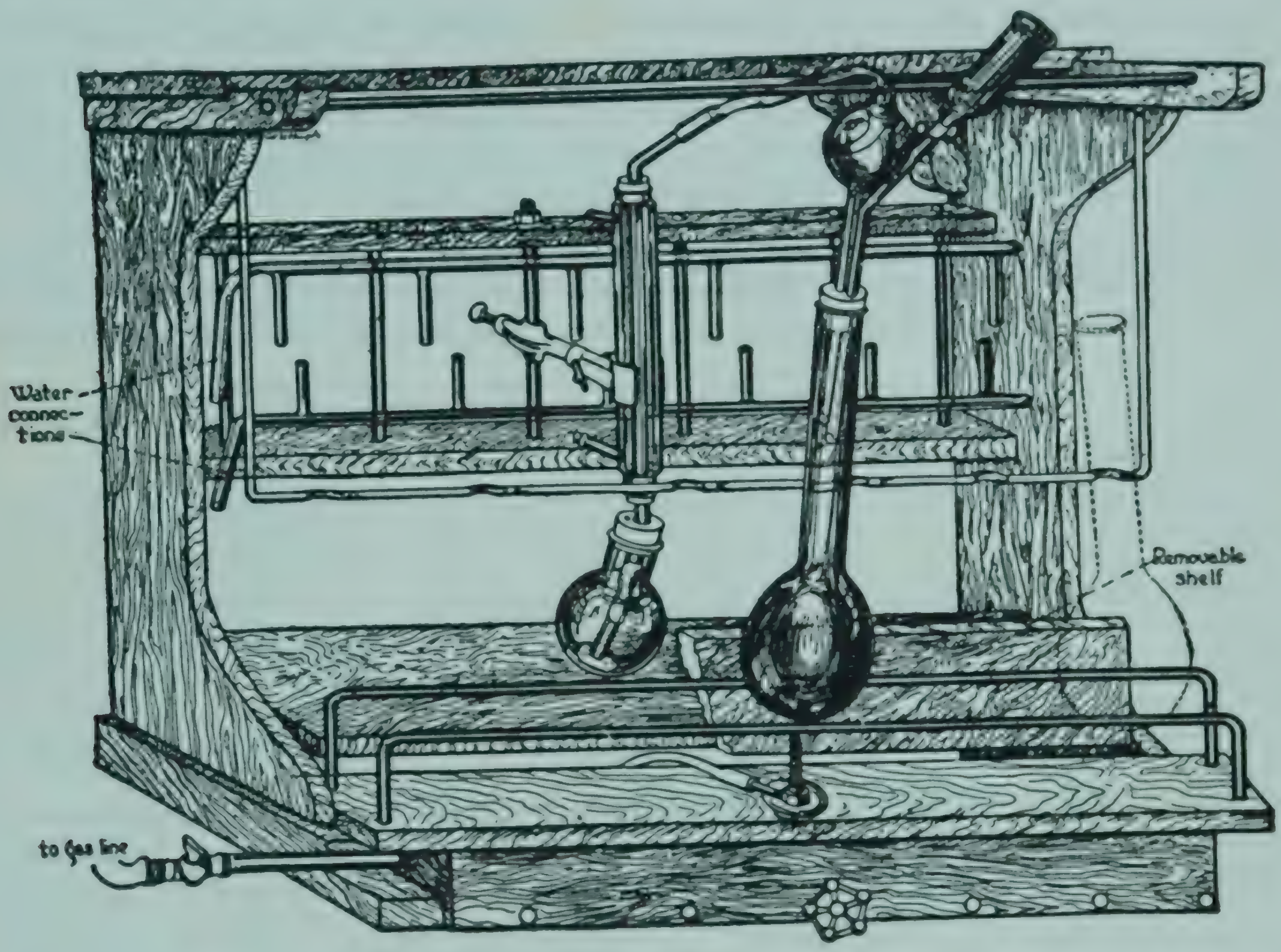

FIG. 251. APPARATUS FOR THE DETERMINATION OF LACTIC ACID (FRIEDEMANN AND GRAESER).

CONJUGATED GLUCURONIC ACIDS

A satisfactory quantitative method which is applicable to the analysis of all conjugated glucuronic acids is not available. Quick[131] has described a method for menthol glucuronic acid in urine which has been extended, with slight modifications, to the determination of other conjugated glucuronic acids. It consists in extraction of the compound with ether, hydrolysis with dilute acid, and finally determination of the liberated glucuronic acid by a copper reduction method. A method for benzoyl glucuronic acid, similar to the above, was reported by Csonka.[132] Maughan, Evelyn, and Browne[133] have described a quantitative photometric version of the naphthoresorcinol test (see p. 843) which appears to give good results.

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[131] Quick: *J. Biol. Chem.*, **61**, 667 (1924); **67**, 477 (1926); **69**, 549 (1926).

[132] Csonka: *J. Biol. Chem.*, **60**, 545 (1924).

GLUCOSE[134]

1. Benedict's Method:[135] ***Principle.*** Benedict's reagent for the estimation of reducing sugars contains potassium thiocyanate as well as copper sulfate, and in the presence of the former a white precipitate of cuprous thiocyanate is formed in reduction instead of the usual red precipitate of cuprous oxide. The small amount of potassium ferrocyanide also aids in keeping cuprous oxide in solution. As the precipitate formed is white the loss of all blue tint in the solution, indicating complete reduction of the copper, is readily observed. The alkali used is sodium carbonate, which has the advantage over hydroxides of being less likely to cause destruction of small amounts of sugar. The solution also has the great advantage of being stable for an indefinite length of time. The method is recommended for its simplicity and accuracy.

Procedure. **The urine, 10 ml. of which should be diluted with water to 100 ml. (unless the sugar content is believed to be low, when it may be used undiluted), is placed in a 50-ml. buret and the volume adjusted to the zero mark. Twenty-five ml. of the reagent[136] is measured with a pipet into a porcelain evaporation dish or casserole (100 to 125 mm. in diameter), 20 g. of crystallized sodium carbonate (or one-half the weight of the anhydrous salt) is added, together with a small quantity of powdered pumice stone or talcum, and the mixture heated to boiling over a free flame and stirred with a glass rod to aid in dissolving the bulk of the carbonate. The diluted urine is now run in from the buret rather rapidly, until a chalk-white precipitate forms in noticeable amount and the blue color of the mixture begins to lessen perceptibly; after which the solution from the buret must be run in a few drops at a time, until the disappearance of the last trace of blue color, which marks the end point. The final color at the end point may be yellow or brown, owing to urinary pigments, but there should be no trace of blue (or green) color. The solution must be kept vigorously boiling and be stirred continuously throughout the entire titration. If the mixture becomes too concentrated during the process, water may be added from time to time to replace the volume lost by evaporation. Any material which dries out on the sides of the dish during the titration must be pushed back into the solution with the stirring rod before the end point is reached.**

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[133] Maughan, Evelyn, and Browne: *J. Biol. Chem.*, **126**, 567 (1938). See also Deichmann: *J. Lab. Clin. Med.*, **28**, 770 (1943).

[134] The method for sugar adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America is given on p. 923.

[135] Benedict: *J. Am. Med. Assoc.*, **57**, 1193 (1911).

[136]

Copper sulfate (crystallized)	18.0 g.
Sodium carbonate (crystallized, one-half the weight of the anhydrous salt may be used)	200.0 g.
Sodium or potassium citrate	200.0 g.
Potassium thiocyanate	125.0 g.
Potassium ferrocyanide (5 per cent solution)	5.0 ml.
Distilled water to make a total volume of	1000.0 ml.

With the aid of heat dissolve the carbonate, citrate, and thiocyanate in enough water to make about 800 ml. of the mixture and filter if necessary. Dissolve the copper sulfate separately in about 100 ml. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five ml. of the reagent are reduced by 50 mg. of glucose.

CALCULATION. The 25 ml. of copper solution is reduced by exactly 50 mg. of glucose. Therefore the volume run out of the buret to effect the reduction contained 50 mg. of the sugar. The formula for calculating the percentage of the sugar is the following: $\frac{0.050}{x} \times D \times 100$ = per cent in original sample, wherein x is the number of ml. of the diluted urine required to reduce 25 ml. of the copper solution, and D is the dilution of the urine (D equals 1 for undiluted urine, 10 for urine diluted 1:10, etc.).

In the use of this method chloroform must not be present during the titration. If used as a preservative in the urine it may be removed by boiling a sample for a few minutes, and then diluting to its original volume.

Interpretation. Glucose in the urine in amounts sufficient to be detected by the commonly employed qualitative tests (i.e., 0.1 to 0.2 per cent or more) ordinarily indicates a pathological condition, although it must be remembered that benign glucosuria is not uncommon (see p. 823 for further discussion), and that other reducing sugars (lactose, pentose) cannot be distinguished from glucose by the ordinary reduction tests, either qualitative or quantitative. Persistent glucosuria may indicate diabetes mellitus, a disorder in which the amount of sugar may rise as high as 10 per cent and averages 3 to 5 per cent. The volume of urine excreted per day is usually also large and the absolute sugar excretion may thus be very great (100 g. of glucose per day are not uncommon). The quantitative methods for the estimation of sugar in urine enable us to determine the severity of this disorder as well as to follow its course under treatment, etc.

2. *Sumner's Method:*[137] ***Principle.*** Urine is heated with a dinitrosalicylic acid reagent which is reduced by the sugar, and the resultant color is compared with standards. This is a rapid method, applicable to normal as well as glycosuric urine, but is not as precise as the more elaborate method of Shaffer and Hartmann described below.

Procedure. **Pipet into a Folin-Wu blood-sugar tube 1 ml. of urine (diluted if necessary) and 3 ml. of the dinitrosalicylic acid reagent.[138] Mix and heat 5 minutes in boiling water. Cool 3 minutes in running water, dilute to 25 ml., mix and compare in a colorimeter with a standard glucose solution treated simultaneously in the same way. A concentration of 0.1 per cent glucose is satisfactory for the standard solution. If the color obtained with the urine is too dark, repeat the test using more dilute urine.**

CALCULATION.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{Dilution}}{10} = \text{per cent glucose}$$

3. *Folin-McEllroy-Peck Method: Principle.* The method is a titration procedure depending upon the use of an alkaline copper solution in which the cupric hydroxide is held in solution by means of phosphate instead of the customary tartrates, citrates, or glycerol. The method is applicable to the determination of lactose in milk.[139]

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[137] Sumner: *J. Biol. Chem.*, **65**, 383 (1925).

[138] To 10 g. crystallized phenol add 22 ml. 10 per cent NaOH. Dissolve in a little water and dilute to 100 ml. Weigh 6.9 g. sodium bisulfite and to this add 69 ml. of alkaline phenol solution. To this add a solution containing 300 ml. 4.5 per cent NaOH, 255 g. Rochelle salt ($NaKC_4H_4O_6 \cdot 4H_2O$), and 880 ml. 1 per cent dinitrosalicylic acid. Mix and keep tightly stoppered in well-filled bottles. The reagent should keep for at least one year. Dinitrosalicylic acid may be obtained from the Eastman Kodak Co., Rochester, N. Y.

4. Method of Shaffer and Hartmann:[140] ***Principle.*** The sugar solution is boiled with Fehling's alkaline copper solution (as modified by Soxhlet) under the conditions prescribed in the standard method of Munson and Walker whose tables may therefore be used in the calculation of results. The residual cupric salt may then be converted into cuprous iodide with the liberation of an equivalent amount of iodine. $2Cu^{++} + 4I^- \rightarrow 2CuI + I_2$. Or the cuprous salt may be oxidized in the presence of a known amount of iodine. $2Cu^+ + I_2 \rightarrow 2Cu^{++} + 2I^-$. Iodine liberated, or excess iodine found in the second case may be titrated with sodium thiosulfate. In the titration of cuprous copper, oxalate is added to depress the ionization of the cupric salt. Shaffer and Hartmann prefer the cuprous titration.

Procedure. **Pipet 25 ml. of each of the two Fehling's solutions[141] into a 300- or 400-ml. flask. Add 50 ml. or less of the (approximately neutral) sugar solution containing from 20 to 200 mg. of sugar, and water (if necessary) to make a total volume of 100 ml. Cover with a small inverted beaker and heat on an asbestos mat over a flame so adjusted as to bring the solution to boiling in 4 minutes. Boil 2 minutes. Stand the flask in the sink under running water till cool (3 to 4 minutes). Then use one of the two following procedures.**

Cuprous Titration. **Add 50 ml. (accurate pipet) or 25 ml. if but little cuprous oxide is present, of iodate-iodide solution,[142] followed by 15 to 17 ml. of 5 N H_2SO_4. The acid should be added from a cylinder or fast-flowing pipet in order that acidification of the whole solution may be accomplished promptly. Shake the solution gently for a few moments until the cuprous oxide has dissolved. The solution should become clear, but some cuprous iodide may separate. Add 20 ml. of saturated solution of potassium oxalate and rotate the flask until the cuprous iodide is *completely* dissolved.**

Titrate with 0.1 N sodium thiosulfate (see Appendix) adding a few ml. of starch solution toward the end before the disappearance of the green color. Run a blank by boiling the Fehling's solution with 50 ml. of water instead of sugar solution.

CALCULATION. From the blank titration subtract the titration of the sugar determination, the remainder representing I_2 required for the oxidation of the cuprous salt. Multiply by the copper factor of the thiosulfate (1 ml. of 0.1 N = 6.36 mg. of Cu), and find the amount of sugar equivalent to the copper by reference to Munson-Walker tables[143] or divide the amount of copper reduced by the corresponding ratio obtained from the glucose curve in Fig. 252.

Cupric Titration. **To the cooled alkaline copper solution add 6 g. of KI and 25 ml. of 5 N H_2SO_4. Titrate with standard thiosulfate, adding starch**

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[139] Folin and Peck: *J. Biol. Chem.*, **38,** 287 (1919).

[140] Shaffer and Hartmann: *J. Biol. Chem.*, **45,** 365 (1921).

[141] *Fehling's Solutions (Soxhlet Modification).* (I) Dissolve 34.64 g. $CuSO_4{\cdot}5H_2O$ in water to make 500 ml. (II) Dissolve 173 g. Rochelle salt and 50 g. NaOH in water to make 500 ml.

[142] *Iodate-Iodide Solution.* Dissolve 5.4 g. KIO_3 and 60 g. KI in water to which a small amount of alkali has been added and dilute to a liter.

[143] *Methods of Analysis of the Association of Official Agricultural Chemists*, 7th ed., Washington, D. C., 1950.

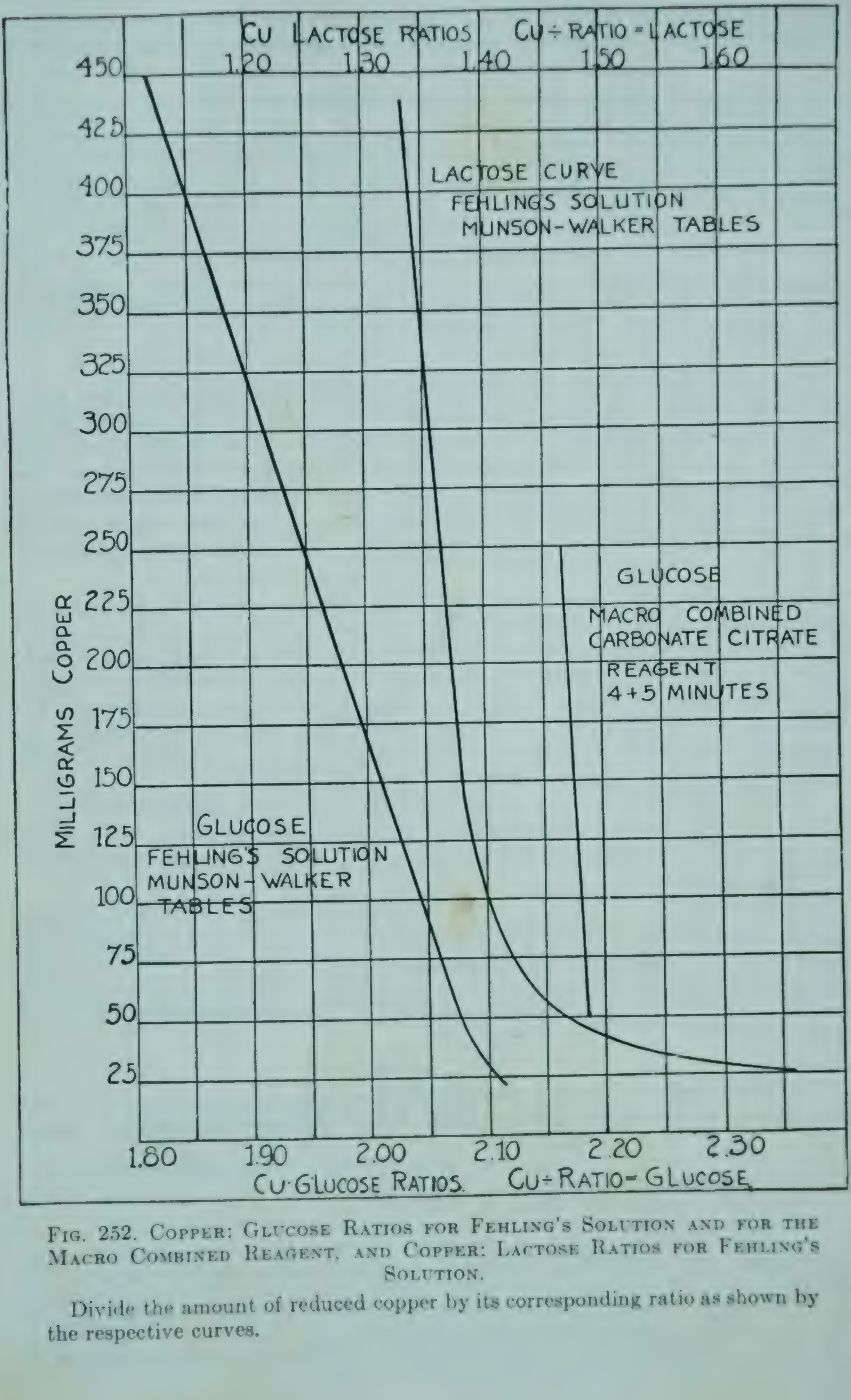

FIG. 252. COPPER: GLUCOSE RATIOS FOR FEHLING'S SOLUTION AND FOR THE MACRO COMBINED REAGENT, AND COPPER: LACTOSE RATIOS FOR FEHLING'S SOLUTION.

Divide the amount of reduced copper by its corresponding ratio as shown by the respective curves.

solution toward the end. The titration is subtracted from a similar blank determination on the Fehling's solution, the difference representing copper reduced by the sugar. For the cupric titration the copper solution must be measured accurately. Consult tables or chart (Fig. 252) for sugar values.

5. Hanes' Modification of the Hagedorn-Jensen Method: Principle. The Hagedorn-Jensen method (see Chapter 23) is modified so that it may be used for larger amounts of glucose (1 to 3 mg.). Maltose may also be determined in the presence of starch. Power and Wilder adapt the method to the determination of 1 to 50 mg. of glucose in urine. For accurate results the urine must be cleared with mercury. For an accuracy within 1 to 5 per cent on diabetic urines the uncleared urine may be used, a correction of 0.25 per cent glucose being subtracted.

Procedure. Into a test tube (1 × 7 in.) pipet 5 ml. of Solution A (alkaline ferricyanide).[144] Add 5 ml. of unknown solution (if necessary add water to make the 5 ml. volume). In another tube put 5 ml. of water and 5 ml. of Solution A. Incline the tube to mix in any drops of liquid adhering to the sides. Cover with glass bulbs (with about an inch of tubing left attached). Place for 15 minutes in a boiling water bath 2 to 3 in. deep. Cool for 3 minutes in cold running water.

Add 5 ml. of Solution B and then 3 ml. of Solution C, using rapid pipets which need not be precise. Run in thiosulfate to a pale yellow color, add a few drops of starch, and titrate to disappearance of the blue color. Subtract titration of unknown from titration of the blank. Convert result into ml. of 0.01 N thiosulfate. Consult Fig. 253 for amounts of glucose or maltose. In the presence of starch stir more vigorously and titrate more slowly. The maltose values are also easily calculated by multiplying the number of ml. of 0.01 N thiosulfate used by 0.414 mg. Invert sugar and thus indirectly sucrose may also be determined. The factor for invert sugar using a final volume of 15 instead of the usual 10 ml. is 0.347 and for sucrose 0.329. For a standard invert sugar solution dissolve 0.95 g. of sucrose in 150 ml. of water, add 30 ml. of 0.5 N HCl, heat to boiling, and boil 1 minute. Cool rapidly, add 30 ml. of 0.5 N NaOH, and dilute to 500 ml. 100 ml. will contain 0.2 g. of invert sugar. Other sugars may also be determined. The factor for fructose is 0.32, for ribose or an equimolecular mixture of glucose and galactose 0.38, arabinose 0.35, mannose and rhamnose 0.34, xylose 0.33, lactose and galactose 0.43.

6. Benedict's Picrate Method (ADOPTED BY THE COMMITTEE ON URINARY IMPAIRMENTS OF THE ASSOCIATION OF LIFE INSURANCE MEDICAL DIRECTORS OF AMERICA): ***Principle.*** The color produced by the reduction of picric acid (p. 67) is compared with permanent inorganic standards representing definite concentrations of sugar.

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[144] *Solution A.* Dissolve 8.25 g. potassium ferricyanide and 10.6 g. anhydrous sodium carbonate in water to make 1000 ml. Store in an opaque bottle and keep 2 to 3 days before use. *Solution B.* Dissolve 12.5 g. KI, 25.0 g. zinc sulfate, and 125 g. NaCl in water to make 500 ml. Traces of iodine appear in the solution on storing. Remove by filtering through 2 thicknesses of filter paper. *Solution C.* Dilute 5 ml. of glacial acetic acid to 100 ml. *Starch.* Stir up 1 g. of Merck soluble starch with 20 ml. water, add to 60 ml. boiling water, boil 2 minutes, cool, and make up to 100 ml. Will keep for several months. *Sodium Thiosulfate.* An approximately N/75 solution used in a 10-ml. buret graduated in 0.02-ml. divisions. Dissolve 3.33 g. of the salt in 10 liters of boiled-out water. Keep in bottle protected by soda-lime tube and run into buret by siphon. Standardize each day at first, then every three to four days. Pipet 5 ml. 0.02 N KIO_3 solution (0.715 g. in 1000 ml.) into a tube. Add 5 ml. 2 per cent KI and 3 ml. 5 per cent acetic acid. Titrate using starch as indicator.

→ 1 Lit.

Procedure. Measure 1 ml. of urine into a test tube graduated at 25 ml. Add 3 ml. of picric acid solution (2 g. of pure dry picric acid per liter) and 0.5 ml. of 5 per cent NaOH. Add next 5 drops of 50 per cent acetone solution (prepared fresh each day by diluting acetone with an equal volume of water) and place the tube promptly in a boiling water bath. In 12 minutes remove the tube, cool, and dilute the contents to 25 ml. Compare this colored solution with the permanent standards in tubes of the same dimensions, and estimate the amount of sugar. The permanent sugar standards are made as described below.

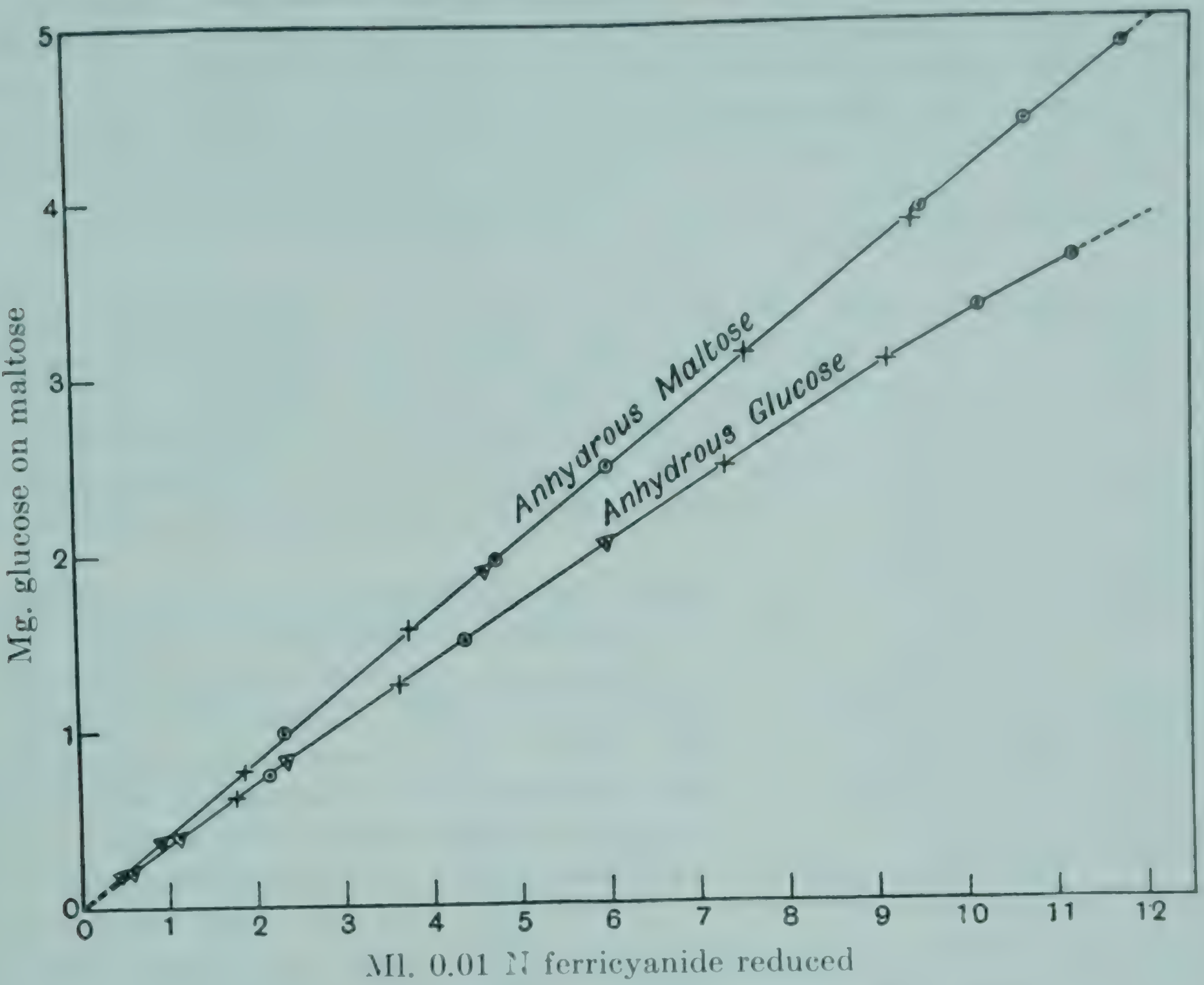

FIG. 253. CHART FOR SUGAR VALUES BY HANES' METHOD.

PERMANENT SUGAR STANDARDS[145]

Put each standard in test tubes of the same diameter as used in sugar determination.

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[145] Solutions needed.

Ferric Chloride (Merck's Analyzed).
Dissolve 200 g. ferric chloride ($FeCl_3 \cdot 6H_2O$) in about 300 ml. distilled water, transfer to a 500-ml. volumetric flask, make up to volume with distilled water, and mix well. Filter through a dry filter paper.

Cobalt Chloride (analyzed grade).
Dissolve 150 g. cobalt chloride ($CoCl_2 \cdot 6H_2O$) in about 300 ml. distilled water, transfer to a 500-ml. volumetric flask, make up to volume with distilled water and mix well. Filter through a dry filter paper.

Dilute Hydrochloric Acid.
Dilute 5 ml. concentrated hydrochloric acid to 50 ml. with distilled water.

Sugar %	*Ferric Chloride Solution* ml.	*Cobalt Chloride Solution* ml.	*Dilute Hydrochloric Acid* ml.	*Water*
0.1	18	7	8	to make 100 ml.
0.2	28	13	8	
0.3	22	22	8	
0.4	16	30	8	
0.5	14	40	8	

7. Hawkins and Van Slyke Method:[146] ***Principle.*** The time required for the urine to decolorize potassium ferricyanide solution is an index of the amount of reducing sugar present. Since normal constituents in concentrated urine may give reduction equivalent to as high as 0.4 per cent glucose, the method is regulated to determine reducing substances in concentrated urines in concentrations of 0.5 per cent and above, and in dilute urine in concentrations of 0.25 per cent and above.

Procedure. **Dilute 1 ml. of urine to 50 ml. If urine is known to be high in sugar (over 3 per cent) dilute 1:100; if low in sugar, dilute 1:25. Albumin need not be removed. Pipet 2 ml. of diluted urine into a pyrex test tube (14 × 125 mm. outside diameter). Add 2 ml. of ferricyanide solution.**[147] **Mix. Immerse in a beaker of boiling water. A similar test tube containing water is immersed for comparison. Make a white background by pasting paper on the side of the beaker away from the observer. A casserole may be used instead of a beaker. Determine the time in seconds required for the last trace of yellow to disappear, preferably using a stop watch. From the chart, Fig. 254, obtain the percentage of sugar in the urine. The chart is for a dilution of 1:50. If a 1:25 dilution is used, divide the result obtained from the chart by 2; for a 1:100 dilution, multiply the result by 2.**

8. Fermentation Method: Principle. This method consists in the measurement of the volume of carbon dioxide evolved when the dextrose of the urine undergoes fermentation with yeast. None of the various methods whose manipulation is based upon this principle is *absolutely* accurate. The method in which Einhorn's saccharometer (Fig. 15, p. 69) is employed is perhaps as satisfactory as any for clinical purposes.

Procedure. **To about 15 ml. of urine in a mortar, add about 0.25 g. of active dry yeast and mix thoroughly. Transfer the mixture to the saccharometer,**

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[146] Hawkins and Van Slyke: *J. Biol. Chem.*, **81**, 459 (1929). For a similar procedure using smaller volumes of solution (and therefore with a slightly different calibration curve), which is also directly applicable to tungstic acid filtrates of blood, see Hawkins: *J. Biol. Chem.*, **84**, 69 (1929). A photometric adaptation of this procedure is described by Hoffman: *J. Biol. Chem.*, **120**, 51 (1937).

[147] *Potassium Ferricyanide Reagent.* Dissolve 75 g. of anhydrous potassium carbonate and 75 g. of potassium bicarbonate in about 750 ml. of water. Dissolve 1 g. of potassium ferricyanide in about 100 ml. of water. Add the ferricyanide solution quantitatively (rinsing with water) to the carbonate-bicarbonate solution in a 1-liter volumetric flask. Dilute to the mark with water and mix. Filter and store in a brown glass-stoppered bottle in the dark. This reagent appears to keep indefinitely. Of the various ingredients, only the ferricyanide need be weighed accurately.

being careful that the graduated tube is completely filled and that no air bubbles gather at the top. Allow the apparatus to stand in a warm place (30° C.) for 12 hours, and observe the percentage of dextrose as indicated by the graduated scale of the instrument. Both the percentage of dextrose and the number of cubic centimeters of carbon dioxide are indicated by the graduations on the side of the saccharometer tube. Controls should be run using normal urine and such urine to which sugar has been added.

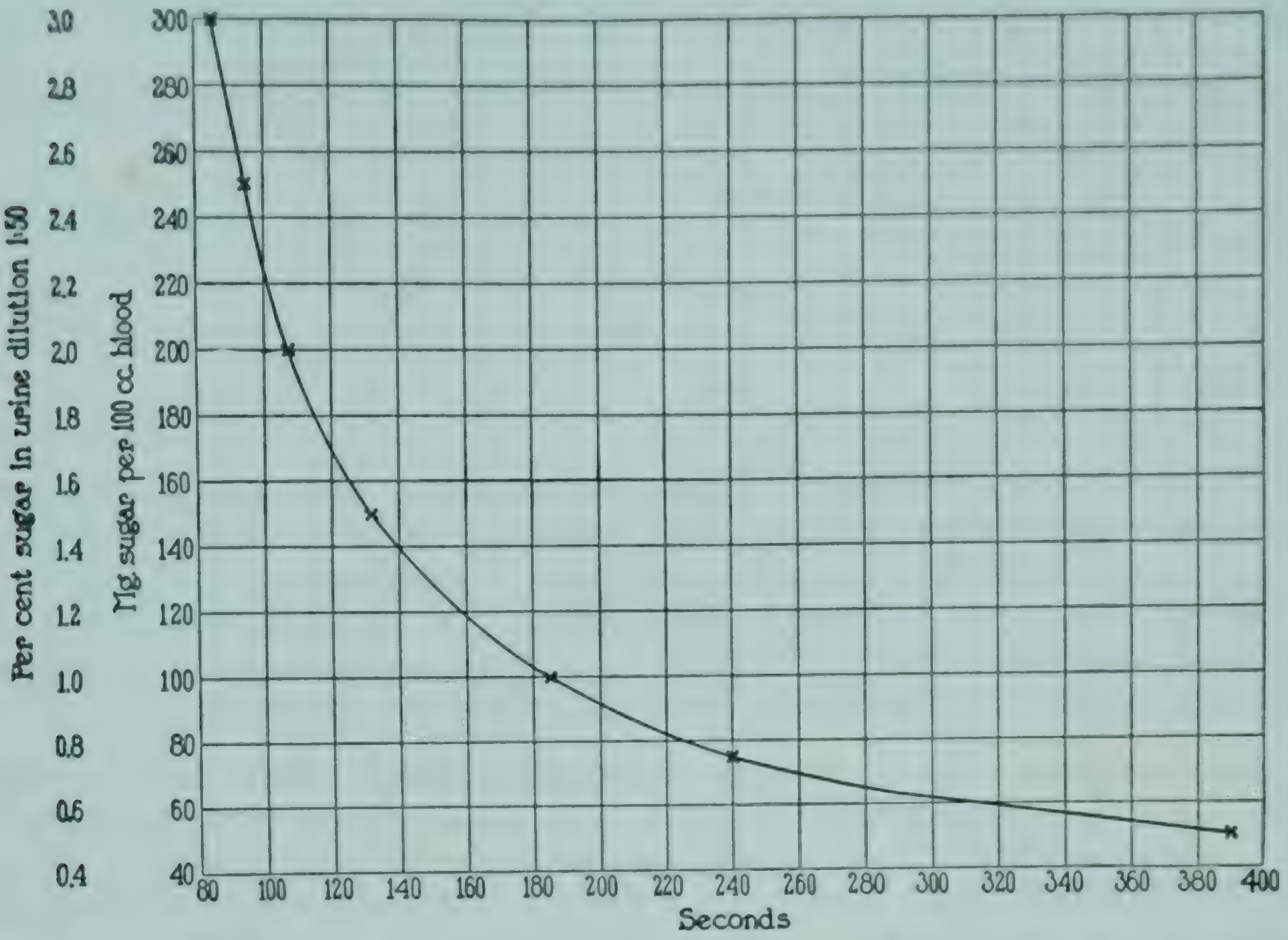

FIG. 254. CHART OF URINE SUGAR VALUES FOR HAWKINS-VAN SLYKE METHOD. *J. Biol. Chem.*, **81**, 459 (1929).

9. Polariscopic Examination. Before subjecting urine to a polariscopic examination, the slightly acid fluid should be decolorized as thoroughly as possible by the addition of a little basic lead acetate. The urine should be well stirred and then filtered through a filter paper which has not been previously moistened. In this way a perfectly clear and almost colorless liquid is obtained.

In determining dextrose by means of the polariscope it should be borne in mind that this carbohydrate is often accompanied by other optically active substances, such as proteins, fructose, β-hydroxybutyric acid, and conjugate glucuronates which may introduce an error into the polariscopic reading; the method is, however, sufficiently accurate for practical purposes.

For directions as to the manipulation of the polariscope, see p. 69.

The table gives the specific rotations of some physiologically important sugars as well as of certain other optically active substances

	Specific Rotation
Glucose	+ 52.49
Maltose	+136.5
Isomaltose	+ 68.0
Lactose	+ 52.53
Fructose	− 92.25
β-Hydroxybutyric acid	− 24.12
Conjugated glucuronic acids	Levorotatory in varying degrees

the possible presence of which must be borne in mind in determining glucose polarimetrically in urine.

DETERMINATION OF SUGAR IN NORMAL URINE

Principle. Since the nature of normal urine "sugar" (i.e., reducing substances) is not definitely established, it is not possible to state which of the methods of determination is to be preferred. The methods involve removal of interfering substances and colorimetric determination of reducing power.

Procedure.

(a) REMOVAL OF INTERFERING SUBSTANCES: METHOD OF FOLIN AND SVEDBERG.[148] **To 5 ml. of urine in a 50-ml. Erlenmeyer flask add 5 ml. of 0.05 N oxalic acid, 10 ml. of water, and (last) 1.5 g. of Lloyd's alkaloidal reagent.[149] Shake gently for 4 minutes. Filter through a quantitative paper into another small flask containing 2 g. of Permutit. Shake 3 minutes. Decant.**

(b) DETERMINATION OF SUGAR. **Sugar may be determined in filtrates from treatment with Lloyd's reagent by one of the methods used for blood sugar[150] (see Chapter 23). The method of Sumner (see p. 920) is said to be applicable to urine without previous treatment. The urine may also be fermented and fermentable sugar determined by measurement of CO_2 given off or by difference. To 10 ml. of the filtrate from treatment with Lloyd's reagent add 3 ml. of a suspension of 1 cake of compressed yeast in 20 ml. of water. Keep at 37° C. for 40 minutes. Filter and determine sugar. Fermentable sugar is usually about 0.01 per cent (see Chapter 28). The urine may also be hydrolyzed and total sugar determined. To 8 ml. of urine filtrate add 1 ml. of 2.6 N H_2SO_4 and heat in a boiling water bath for 75 minutes. Add 1 ml. of silica-free NaOH exactly equivalent to the acid added. Determine sugar by any of above methods.**

PROTEIN[151]

1. Colorimetric Determination of Proteins (Method of Hiller, McIntosh, and Van Slyke):[152] ***Principle.*** The protein is precipitated with trichloroacetic acid,[153] dissolved in NaOH solution, and copper ions added to give a biuret color which is compared with a standard.

Procedure. **Measure 2 ml. of the urine (previously adjusted to a pH of about 7.4) into a centrifuge tube, add an equal volume of 10 per cent trichloroacetic acid solution, mix and centrifuge for 5 minutes. If the volume of the precipitate is less than 0.2 or more than 0.6 ml., repeat using more or less urine. Pour off the supernatant fluid. Dissolve the precipitate in about 3 ml. of 3 per cent NaOH and wash into a tube graduated at 10 ml. with portions of the 3 per cent NaOH until the volume is about 9 ml. Add 0.25 ml. of 20 per cent**

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[148] Folin and Svedberg: *J. Biol. Chem.*, **70**, 405 (1926). Hamilton (*J. Biol. Chem.*, **78**, 63 (1928)) omits Permutit but uses Lloyd's reagent extracted with concentrated HCl for 1 day, washed with water, and then extracted with concentrated HNO_3 for one day.

[149] Obtainable from Eli Lilly and Co., Indianapolis, Ind.

[150] See Folin: *J. Biol. Chem.*, **70**, 405 (1926); Benedict: *J. Biol. Chem.*, **68**, 766 (1926).

[151] The method for albumin adopted by the Committee on Urinary Impairments of the Association of Life Insurance Medical Directors of America is given on p. 929.

[152] Hiller, McIntosh, and Van Slyke: *J. Clin. Invest.*, **4**, 235 (1927).

[153] According to Beckman, Hiller, Shedlovsky, and Archibald (*J. Biol. Chem.*, **148**, 247 (1943)), from 4 to 20 per cent of the protein in proteinuria may not be precipitable by trichloroacetic acid. This may account for discrepancies in protein determination between methods involving trichloroacetic acid precipitation and those not involving such precipitation.

copper sulfate solution and dilute to 10 ml. with the NaOH. Mix by shaking. Let stand 10 minutes. Centrifuge and compare with a standard. For a standard measure 5 ml. of a biuret solution containing 13.33 mg. of biuret, equivalent to 12.3 mg. of protein, into a tube graduated at 10 ml. Add distilled water to 8 ml., 1 ml. of 30 per cent NaOH, 0.25 ml. of copper sulfate, and water to make 10 ml. Mix, let stand for 10 minutes, centrifuge, and compare in a colorimeter with the unknown, setting this standard at 15 mm.

For determining albumin add to 10 ml. of the urine, at pH 7.4, 10 ml. of 44 per cent sodium sulfate solution, mix well, and put in an incubator at 37° C. for three hours. Filter. Proceed as for total protein, using four times the volume. The globulin is estimated by difference.

CALCULATION. *For total protein:*

$$\frac{15}{R} \times \frac{12.3}{\text{ml. urine used}} = \text{g. protein per liter}$$

For albumin:

$$\frac{15}{R} \times \frac{12.3 \times 2}{\text{ml. filtrate used}} = \text{g. albumin per liter}$$

2. *Folin's Gravimetric Method for the Determination of Protein in Urine: Principle.* The protein precipitated by heat and acetic acid is centrifuged, washed, dried, and weighed.

Procedure. Pipet 10 ml. of urine into an ordinary conical centrifuge tube, which has been previously weighed; add 1 ml. of 5 per cent acetic acid, and let stand for 15 minutes in a beaker of boiling water. At the end of this time remove the tube from the water bath, and centrifuge for a few minutes. Pour off the supernatant liquid, stir up the precipitate in the tube with about 10 ml. of boiling 0.5 per cent acetic acid, and again centrifuge. Remove the supernatant liquid from the precipitate in the tube, and wash once more, this time with 50 per cent alcohol. After centrifuging, pour off the supernatant alcohol and place the tube for 2 hours in an air bath at 100 to 110° C.; then cool in a desiccator, and weigh.

The precipitate may also be filtered off and washed on a small tared filter paper, then dried and weighed. In this case it is better to use a larger volume of urine. Instead of weighing, the nitrogen in the precipitate may be determined by the Kjeldahl method.[154]

CALCULATION. Multiply the weight of the precipitate in grams by 10 to obtain the percentage of protein present. If a volume other than 10 ml. of urine is used multiply by the appropriate volume factor.

Interpretation. The amount of albumin occurring in the urine is not necessarily an index of the severity or type of the disorder giving rise to it. Hence no significant figures can be given. Normal human urine probably contains a trace of albumin which is too slight to be detected or determined by the usual procedures. The determination of albumin may be of assistance in following the course of kidney disturbances, but the results can be interpreted only in the light of other clinical findings. (See discussion under "Albumin," p. 828.)

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[154] In order to arrive at correct figures for the protein content, it is then only necessary to multiply the total nitrogen content by 6.25 (see p. 239). Correction should be made for the nitrogen content of the filter paper used unless this factor is negligible.

3. Esbach's Method: Principle. This method depends upon the precipitation of protein and measurement of its volume after settling. The apparatus used is Esbach's albuminometer (Fig. 255).

***Procedure.* In making a determination fill the albuminometer to the point *U* with urine, then introduce Esbach's reagent[155] (or 10 per cent trichloroacetic acid, as recommended by Quick) until the point *R* is reached. Now stopper the tube, invert it slowly several times in order to insure the thorough mixing of the fluids, and stand the tube aside for 24 hours. Creatinine, resin, acids, etc. are precipitated in this method, and for this and other reasons it is not so accurate as the coagulation method. It is, however, extensively used clinically. According to Sahli, Esbach's method is "accurate approximately to 1 part per 1000," whereas Pfeiffer claims it is not accurate for less than one-half or for more than 5 parts per 1000.**

CALCULATION. The graduations indicate g. of protein per liter of urine. Thus a reading of 3 indicates 3 g. per liter, or 0.3 per cent protein.

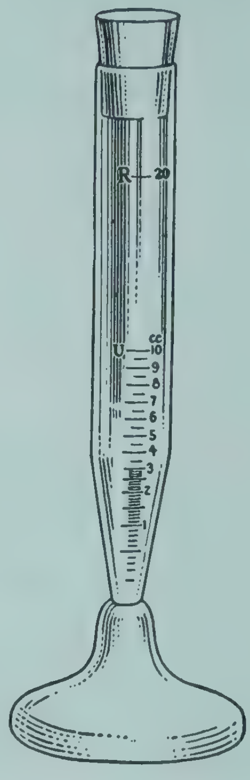

FIG. 255. ESBACH'S ALBUMINOMETER.

4. The Determination of Albumin (METHOD ADOPTED BY THE COMMITTEE ON URINARY IMPAIRMENTS OF THE ASSOCIATION OF LIFE INSURANCE MEDICAL DIRECTORS OF AMERICA): ***Principle.*** Clarified urine is treated with sulfosalicylic acid and the degree of turbidity produced is measured by comparison with artificial standards.

***Procedure.* Pipet 2.5 ml. of centrifuged urine into a test tube graduated at 10 ml. and add 3 per cent sulfosalicylic acid (30 g. in 1000 ml. of distilled water) to the 10-ml. mark. Invert the tube to mix, allow to stand 10 minutes, and compare the turbidity with the permanent turbidity standards. Record the value of the standard most closely matched, as the albumin content of the urine.**

***Preparation of Permanent Albumin Standards.*[156] Dissolve 20 g. of gelatin (Super X brand in sheet form, Coignet Chemical Products Co., New York) in 120 to 140 ml. of distilled water at 45° to 55° C. and make up to 200 ml. Add about half the white of an egg and stir in. Heat in a water bath for at least 30 minutes after a temperature of 90° C. has been attained. Filter hot through a Whatman's No. 4 paper yielding a perfectly clear, slightly yellow solution. Immediately before using, add 0.3 ml. of formalin (40 per cent formaldehyde solution) to each 100 ml. of gelatin solution. Formazin, the material to be suspended in the gelatin, is made as follows: Dissolve 2.5 g. of urotropin in 25 ml. of distilled water at room temperature. Add to 25 ml. of 1 per cent hydrazine sulfate solution also at room temperature. Mix, stopper, and allow to stand at least 15 hours. Suspend the white amorphous precipitate uniformly by gently inverting the flask a few times. Add 14.5 ml. of the formazin suspension to 100 ml. of 10 per cent gelatin solution at**

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[155] See Appendix.

[156] These permanent standards were developed in the laboratories of the Metropolitan Life Insurance Company and the Mutual Benefit Life Insurance Company. For a detailed description of the preparation of these standards, see Kingsbury, Clark, Williams, and Post: *J. Lab. Clin. Med.*, **11**, 981 (1926).

45° to 55° C. (to which the correct amount of formalin has been added) and mix thoroughly. This produces a turbidity equivalent to that made by an albumin solution of 0.1 per cent, or one containing 100 mg. of albumin in 100 ml. when precipitated by 3 volumes of 3 per cent sulfosalicylic acid. Dilute this stock suspension as follows to make the remaining standards:

Stock Formazin Suspension Equivalent to 100 mg. Albumin per 100 ml.	*10 % Clarified Gelatin*	*Value of Standard Made*	
ml.	*ml.*	%	*mg.*
25.0	26	0.05	50
20.0	30	0.04	40
15.0	35	0.03	30
10.0	40	0.02	20
5.0	45	0.01	10
2.5	55	0.005	5

Pour each standard into a test tube of the same dimensions as those used in making the test with urine. Seal the tube with a waxed stopper and allow to cool to room temperature. In a short time the gelatin should solidify and after a few days cannot be melted at any room temperature. In extremely hot weather put the tubes in a cool place for a few days before any attempt is made to use them. Keep the standards in a well-lighted room. If in time they become greenish, they may be bleached by exposure to sunlight without changing their turbidimetric value. The standards may be checked against sheep serum standards of known protein content precipitated in the same manner as in the urine test. Standards older than 8 months should be replaced unless actual tests show that they have their original degree of turbidity. In the course of a year there may be only a very slight diminution in the turbidimetric value of the standards. There is no detectable change in 6 to 8 months.

ACETONE BODIES

1. Van Slyke's Methods:[157] ***Principle.*** The method is based on a combination of Shaffer's oxidation of β-hydroxybutyric acid to acetone (p. 934), and Denigès' precipitation of acetone as a basic mercuric sulfate compound. Glucose and certain other interfering substances are removed by precipitation with copper sulfate and calcium hydroxide. Preservatives other than toluene or copper sulfate should not be used.

Procedure: Removal of Glucose and Other Interfering Substances from Urine. **Place 25 ml. of urine in a 250-ml. measuring flask. Add 100 ml. of water, 50 ml. of copper sulfate solution,**[158] **and mix. Then add 50 ml. of 10 per cent**

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[157] Van Slyke: *J. Biol. Chem.*, **32**, 455 (1917); **83**, 415 (1929).

[158] Solutions Required. *20 Per Cent Copper Sulfate.* Dissolve 200 g. $CuSO_4 \cdot 5H_2O$ in water and make up to 1 liter.

10 Per Cent Mercuric Sulfate. Dissolve 73 g. pure red mercuric oxide in 1 liter of 4 N H_2SO_4.

50 Volume Per Cent Sulfuric Acid. Dilute 500 ml. sulfuric acid of specific gravity 1.835 to 1 liter with water. Concentration of H_2SO_4 must be readjusted if necessary to make it 17.0 N by titration.

10 Per Cent Calcium Hydroxide Suspension. Mix 100 g. Merck's fine light reagent $Ca(OH)_2$ with 1 liter of water.

5 Per Cent Potassium Bichromate. 50 g. $K_2Cr_2O_7$ dissolved in water and made up to 1 liter.

Combined Reagents for Total Acetone Body Determination. 1 liter of the above 50 per cent sulfuric acid, 3.5 liters of the mercuric sulfate, 10 liters of water.

calcium hydroxide suspension, shake, and test with litmus. If not alkaline, add more calcium hydroxide. Dilute to the mark and let stand at least one-half hour for glucose to precipitate. Filter through a dry folded filter. This procedure will remove up to 8 per cent of glucose. Urine containing more should be diluted enough to bring the glucose down to 8 per cent. The copper treatment is depended upon to remove interfering substances other than glucose, and should therefore never be omitted, even when glucose is absent. The filtrate may be tested for glucose by boiling a little in a test tube. A precipitate of yellow cuprous oxide will be obtained if the removal has not been complete. A slight precipitate of white calcium salts always forms, but does not interfere with the detection of the yellow cuprous oxide.

Determination of Total Acetone Bodies (Acetone, Acetoacetic Acid, and β-Hydroxybutyric Acid). **Place in a 500-ml. Erlenmeyer flask 25 ml. of urine filtrate. Add 100 ml. of water, 10 ml. of 50 per cent sulfuric acid, and 35 ml. of the 10 per cent mercuric sulfate. Or, in place of adding the water and reagents separately, add 145 ml. of the combined reagents. Connect the flask with a reflux condenser having a straight condensing tube of 8 or 10 mm. diameter, and heat to boiling. After boiling has begun, add 5 ml. of the 5 per cent bichromate through the condenser tube. Continue boiling gently 1½ hours. The yellow precipitate which forms consists of the mercury sulfate–chromate compound[159] of acetone (total). It is collected in a Gooch or "medium density" alundum or fritted glass crucible, washed with 200 ml. of cold water, and dried for an hour at 110° C. The crucible is allowed to cool in room air (a desiccator is unnecessary and undesirable) and weighed. Several precipitates may be collected, one above the other, without cleaning the crucible. As an alternative to weighing, the precipitate may be dissolved and titrated as described below.**

Determination of Acetone and Acetoacetic Acid. **The acetone plus the acetoacetic acid, which completely decomposes into acetone and CO_2 on heating, is determined without the β-hydroxybutyric acid exactly as the total acetone bodies, except that (1) no bichromate is added to oxidize the β-hydroxybutyric acid and (2) the boiling must continue for not less than 30 nor more than 45 minutes. Boiling for more than 45 minutes splits off a little acetone from β-hydroxybutyric acid even in the absence of chromic acid.[160]**

Determination of β-Hydroxybutyric Acid. **The β-hydroxybutyric acid alone is determined exactly as total acetone bodies except that the preformed acetone and that from the acetoacetic acid are first boiled off. To do this**

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[159] This contains about 77 per cent mercury and in the absence of chromate has approximately one of the following formulas: $3HgSO_4 \cdot 5HgO \cdot 2(CH_3)_2CO$ or $2HgSO_4 \cdot 3HgO \cdot (CH_3)_2CO$.

[160] *Blank Determination of Precipitate from Substances in Urine Other than the Acetone Bodies.* The 25 ml. aliquot of urine filtrate is treated with sulfuric acid and water and boiled 10 minutes to drive off acetone. The residue is made up to 175 ml. with the same amounts of mercuric sulfate and sulfuric acid used in the above determinations, but without chromate, and is boiled under the reflux for 45 minutes. Longer boiling splits off some acetone from β-hydroxybutyric acid, and must therefore be avoided. The weight of precipitate obtained may be subtracted from that obtained in the above determination.

The blank is so small that it appears to be significant only when compared with the small amounts of acetone bodies found in normal or nearly normal urines. In routine analyses of diabetic urines it is not determined.

Tests of Reagents. When the complete total acetone bodies determination, including the preliminary copper sulfate treatment, is performed on a sample of distilled water instead of urine, no precipitate whatever should be obtained. This test must not be omitted.

the 25 ml. of urine filtrate plus 100 ml. of water are treated with 2 ml. of the 50 per cent sulfuric acid and boiled in the open flask for 10 minutes. The volume of solution left in the flask is measured in a cylinder. The solution is returned to the flask, and the cylinder washed with enough water to replace that boiled off and restore the volume of the solution to 127 ml. Then 8 ml. of the 50 per cent sulfuric acid and 35 ml. of mercuric sulfate are added. The flask is connected under the condenser and the determination is continued as described for total acetone bodies.

Titration of the Precipitate in the Above Methods. Instead of weighing the precipitate, one may wash the contents of the Gooch, including the asbestos, into a small beaker with as little water as possible, and add 15 ml. of normal HCl. The mixture is then heated, and the precipitate quickly dissolves. In case an alundum or glass crucible is used, it is set into the beaker of acid until the precipitate dissolves, and then washed with suction, the washings being added to the beaker. In place of using either a Gooch or alundum crucible, one may, when titration is employed, wash the precipitate without suction on a small quantitative filter paper, which is transferred with the precipitate to the beaker and broken up with a rod in 15 ml. of normal HCl.

In order to obtain a good end point in the subsequent titration it is necessary to reduce the acidity of the solution. For this purpose it has been found that the addition of excess sodium acetate is the most satisfactory means. Six to 7 ml. of 3 M acetate are added to the cooled solution of redissolved precipitate. Then the 0.2 M KI is run in rapidly from a buret with constant stirring. If more than a small amount of mercury is present, a red precipitate of HgI_2 at once forms, and redissolves as soon as 2 or 3 ml. of KI in excess of the amount required to form the soluble K_2HgI_4 has been added. If only a few mg. of mercury are present, the excess of KI may be added before the HgI_2 has had time to precipitate so that the titrated solution remains clear. In this case not less than 5 ml. of the 0.2 M KI are added, since it has been found that the final titration is not satisfactory if less is present. The excess of KI is titrated back by adding 0.05 M $HgCl_2$ from another buret until a permanent red precipitate forms. Since the reaction involved is $HgCl_2 + 4KI = K_2HgI_4 + 2KCl$, 1 ml. of 0.05 M $HgCl_2$ is equivalent in the titration to 1 ml. of the 0.2 M KI.

In preparing the two standard solutions the 0.05 M $HgCl_2$ is standardized by the sulfide method, and the iodine is standardized by titration against it. A slight error appears to be introduced if the iodide solution is gravimetrically standardized and used for checking the mercury solution, instead of vice versa.

In standardizing the mercuric chloride the following procedure has been found convenient: 25 ml. of 0.05 M $HgCl_2$ is measured with a calibrated pipet, diluted to about 100 ml., and H_2S is run in until the black precipitate flocculates and leaves a clear solution. The HgS, collected in a Gooch crucible and dried at 110° C., should weigh 0.2908 g. if the solution is accurate.

Both by gravimetric analyses of the basic mercuric sulfate acetone precipitate and by titration, the mercury content of the precipitate has been found to average 76.9 per cent. On this basis, each ml. of 0.2 M KI solution, being equivalent to 10.0 mg. of Hg, is equivalent to 13.0 mg. of the mercury acetone precipitate.

Titration is not quite so accurate as weighing, but, except when the amounts determined are very small, the titration is satisfactory.

CALCULATION. One mg. of β-hydroxybutyric acid yields 8.45 mg. of precipitate. One mg. of acetone yields 20.0 mg. of precipitate. One ml. of 0.2 M KI solution is equivalent to 13 mg. of precipitate in titration of the latter.

In order to calculate the acetone bodies as β-hydroxybutyric acid rather than acetone, use the above factors multiplied by the ratio of the molecular weights $\frac{\beta\text{-acid}}{\text{acetone}} = \frac{104}{58} = 1.793$. In order to calculate the acetone bodies in terms of molecular concentration, divide the factors in the table by 58. To calculate ml. of 0.1 M acetone bodies per liter of urine, use the above factors multiplied by 10,000/58 or 172.4.

SPECIAL FACTORS FOR CALCULATION OF RESULTS WHEN 25 ML. OF URINE FILTRATE, EQUIVALENT TO 2.5 ML. OF URINE, ARE USED FOR THE DETERMINATION

Determination Performed	*Acetone Bodies, Calculated as g. of Acetone per Liter of Urine, Indicated by:*	
	1 g. of precipitate	*1 ml. of 0.2 M KI sol.*
Total acetone bodies*..................	24.8	0.322
β-Hydroxybutyric acid..................	26.4	0.344
Acetone + acetoacetic acid.............	20.0	0.260

* The "total acetone bodies" factor is calculated on the assumption that the molecular proportion of them in the form β-hydroxybutyric acid is 75 per cent of the total, which proportion is usually approximated in acetonuria. Because β-hydroxybutyric acid yields only 0.75 molecule of acetone, the factors are strictly accurate only when this proportion is present, but the error introduced by the use of the approximate factors is for ordinary purposes not serious. The actual errors in percentage of the amounts determined are as follows: molecular proportions of acetone bodies as β-acid 0.50, error 6.5 per cent; β-acid 0.90, error 3.8 per cent; β-acid 0.80, error 1.3 per cent.

Interpretation. Normal adults on a mixed diet excrete on the average 3 to 15 mg. of combined acetone and acetoacetic acid per day, and anything over 20 mg. is usually pathological. Usually about one-fourth of this total is acetone, although the proportion varies considerably. The amount is considerably increased in fasting and on a carbohydrate-free diet due to the development of ketosis. In severe diabetic ketosis values up to 6 g. per day or even higher may be noted. It is sometimes found in large amounts in intoxications associated with pregnancy. It may be found in increased amounts in the urine in a great variety of pathological conditions. Quantitative estimation enables us to follow the course of the ketosis. Ammonia excretion is also largely increased in these conditions, being used in the neutralization of the excess acids formed in the body. (See also Chapter 29.)

β-Hydroxybutyric acid may occur in normal human urine to the extent of 20 to 30 mg. per day. In fasting or on a carbohydrate-free diet, very large amounts may be excreted (up to 20 g. per day). In severe diabetes mellitus the largest amounts are found, and excretions of 50 or even 100 g. or over per day have been noted. In this condition it is usually the most abundant of the acetone bodies making up from 60 to 80 per cent of the total. The ratio is, however, by no means constant, and it should be borne in mind that in rare cases large amounts of β-hydroxybutyric acid may be eliminated although the acetone excretion is very low. It is always present in the urine when large amounts of acetone are present.

A discussion of disturbance of acid-base equilibrium due to abnormal fat metabolism may be found on pp. 692 and 693.

2. Method of Shaffer and Marriott:[161] ***Principle.*** By this procedure the combined acetone and acetoacetic acid is determined in the same sample of urine used in the determination of β-hydroxybutyric acid. The preformed acetone and the acetoacetic acid are distilled off together as acetone and determined by the iodine titration method. The β-hydroxybutyric acid remains in the residue from distillation and is oxidized by means of potassium bichromate. The product of the oxidation is acetone which is distilled off and determined as such.

Procedure: Determination of Acetone and Acetoacetic Acid. **Measure from 25 to 100 ml.[162] or more of urine (usually 50 ml.) with a pipet into a 500-ml. volumetric flask containing 200 to 300 ml. of water. Add basic lead acetate solution (U.S.P.) in volume equal to that of the urine used[163] and mix well. Add concentrated ammonium hydroxide in amount equal to about one-half that of the lead acetate solution. Dilute the contents of the flask to the mark with water, shake, and let stand for a few minutes. Then filter the liquid, preferably through a folded filter. Measure 200 ml. of the filtrate into a round-bottomed flask (800-ml. or liter Kjeldahl flasks are convenient), dilute with water to about 600 ml., and add 15 ml. of concentrated sulfuric acid and a little talc or a boiling stone. Distil until about 200 ml. of distillate have been collected. The tube of the condenser should dip beneath the surface of the water in the receiving flask so that no loss of acetone will occur. The distilling flask must also be fitted with a dropping tube or dropping funnel so water may be run in from time to time and the volume of liquid in the flask kept from becoming less than 400 to 500 ml. A good condenser should be used, but it is not necessary to cool the distillate in ice.**

The distillate thus obtained is transferred to a second Kjeldahl flask and 10 ml. of 10 per cent NaOH added. It is then redistilled for about 20 minutes.[164] The second distillate is then titrated with standard iodine and thiosulfate solutions.

This is done by adding 10 to 25 ml. of 0.1 N iodine solution (0.02 N if the amount of acetone bodies as indicated by a qualitative test is small) and 10 ml. of strong NaOH (about 40 per cent). Let stand for 10 minutes. Add 18 ml. of concentrated HCl. Titrate with 0.1 N sodium thiosulfate solution to a pale yellow color, add a few ml. of soluble starch solution (see Appendix) and continue titration to disappearance of the blue color.

CALCULATION. Subtract the number of ml. of 0.1 N thiosulfate solution used from the volume of 0.1 N iodine solution employed. Since 1 ml. of the iodine solution is equivalent to 0.967 mg. of acetone, and since 1 ml. of the thiosulfate solution is equivalent to 1 ml. of the iodine solution, if we multiply the remainder from the above subtraction by 0.967 we will obtain the number of mg. of acetone and acetoacetic acid, expressed as acetone, in the volume of urine taken for analysis.

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[161] Shaffer and Marriott: *J. Biol. Chem.*, **16**, 265 (1915).

[162] The amount used depends upon the expected yield of β-hydroxybutyric acid. In urines which give a strong ferric chloride reaction for acetoacetic acid, or when 5 to 10 g. or more of β-hydroxybutyric acid are expected, it is unnecessary to use more than 25 to 50 ml. of urine. However, in case only a trace of β-hydroxybutyric acid is expected, the volume should be much larger as indicated. Under all conditions, the amount specified is sufficient for duplicate determinations. It is desirable to use such a volume of urine as contains the proper amount of β-hydroxybutyric acid to yield 25 to 50 mg. of acetone.

[163] If the urine contains little or no sugar, only half the amount or less of lead acetate should be used.

[164] In many instances when a high degree of accuracy is not required, this redistillation may be omitted and the first distillate titrated directly. The results so obtained are slightly higher than those after redistillation from alkali. The object of the redistillation is to get rid of fatty acids, of which formic acid is one of the most troublesome.

Determination of the β-Hydroxybutyric Acid. **The flask containing the residue from the first distillation above is used in the determination of the β-hydroxybutyric acid. A new receiver is arranged as before with the tip from the condenser dipping beneath the surface of the water. The distillation is then continued and water added whenever necessary to keep the volume between 400 and 600 ml. A dilute solution (1 per cent) of potassium bichromate is added during the distillation. At first 20 ml. of this 1 per cent solution are added slowly through the dropping tube and then 10-ml. portions every 15 to 20 minutes until the whole has been added.[165] Should the liquid become markedly green the bichromate must be added at correspondingly shorter intervals and in amount sufficient to maintain a slight red-yellow color of the chromic acid which may be detected even in the presence of the green. Continue the distillation with moderate boiling for from 2 to 3 hours. The distillate which should be collected in a liter flask to avoid transference is again distilled for about 20 minutes after adding 10 ml. of 10 per cent sodium hydroxide and 25 ml. of 3 per cent hydrogen peroxide. The flask must be heated cautiously until the peroxide has been decomposed. This final distillate is titrated with standard iodine and thiosulfate solutions in the usual manner and the result expressed as hydroxybutyric acid. One ml. of 0.1 N iodine solution is equivalent to 1.736 mg. of hydroxybutyric acid. About 10 per cent should be added to the results for β-hydroxybutyric acid as obtained by this method as the yield of acetone is only about 90 per cent of the theoretical. This error appears to be practically constant, so that satisfactory results may be obtained by correction.**

3. Method of Behre and Benedict, Modified by Behre:[166] ***Principle.*** The acetone, preformed, from acetoacetic acid, and (after removal of interfering substances) from β-hydroxybutyric acid, is distilled from acid solution, and determined colorimetrically or photometrically by its reaction with salicylic aldehyde in alkaline solution. The colored product is dihydroxydibenzene acetone.

Procedure.[167]

Determination of Acetone and Acetoacetic Acid, but Not β-Hydroxybutyric Acid. **To a 200- to 300-ml. distilling flask transfer a measured volume of urine (usually 15 to 30 ml.), and add 3 to 4 drops of 1:1 sulfuric acid. Add a few glass beads and connect immediately to a 200-mm. water-cooled con-**

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[165] From 0.5 g. to 1 g. of bichromate will usually be sufficient, and not more than 1 g. should be added unless the liquid turns green, indicating a great reduction to chromium sulfate. Very rarely 2 or 3 g. of bichromate may be necessary, especially if the sugar has not been completely removed.

[166] Behre and Benedict: *J. Biol. Chem.*, **70**, 487 (1926); Behre: *ibid.*, **136**, 25 (1940).

[167] Reagents Required: *Sulfuric Acid, 1:1.* Carefully pour 500 ml. concentrated sulfuric acid into 500 ml. water, with stirring. Cool, dilute to 1 liter, and mix.

Salicylic Aldehyde. Eastman's technical grade, or Eimer and Amend's "Acid Salicylous, Synthetic" are satisfactory.

Saturated Potassium Hydroxide Solution. Add an excess of solid reagent-grade potassium hydroxide to some water in a beaker. Stir to dissolve as completely as possible, allow to settle, and use the clear supernatant solution. The specific gravity should be 1.540.

Aqueous Alcohol. A 70 to 75 per cent solution of ethyl alcohol in water. Dilute 75 ml. 95 per cent ethyl alcohol to 100 ml. with water, and mix.

Standard Acetone Solutions. Dilute 5 ml. of freshly opened or well-kept c.p. acetone to 500 ml. with water, and mix. For approximate purposes, this may be assumed to produce a 0.78 to 0.79 per cent solution. For accurate work it should be standardized by iodimetric titration. Place 5 ml. acetone solution in a 250-ml. glass-stoppered flask, and add 25 ml. of normal sodium hydroxide solution, followed by 50 ml. (accurately measured) of 0.1 normal iodine solution. Add the iodine solution a little at a time, with mixing. Stopper and allow to stand 10 to 15 minutes. Add 25 to 30 ml. of normal sulfuric acid (there must denser fitted with a delivery tube drawn out to a fine tip. An all-glass apparatus is preferred, the joints being lubricated with water; if this is not available, connections may be made with well-fitting cork (not rubber) stoppers. As receiver for the distillate, use a 15-ml. graduated centrifuge tube, so arranged that the enlarged portion of the delivery tube rests on the rim of the centrifuge tube, acting as a cover for it, and the fine tip of the delivery tube reaches just to the bottom of the receiver. Place a minimal amount of water in the receiver, to cover the tip outlet. Apply heat with a microburner to the contents of the distilling flask, slowly at first to prevent excessive bubbling in the receiver, and distil over a volume of distillate equal to one-third or more of the original volume. Remove the receiver, rinsing off the tip of the delivery tube with a little water in the process, measure the total volume in the receiver, or dilute with water to a definite volume, and mix by inversion.

For color development, transfer 0.1 ml. of salicylic aldehyde to a test tube graduated at 5 and 10 ml. Add 2 ml. of distillate, followed by 1.5 ml. of saturated potassium hydroxide solution from a buret with a fine glass tip. Mix the contents of the tube by several churning motions with a footed glass rod, leave the rod in the tube, and allow to stand 20 minutes at room temperature. Finally add either (*A*) water to the 10-ml. mark, or (*B*) aqueous alcohol to the 5-ml. mark, rinsing and removing the rod in the process. Mix by tapping or inversion. Choice between procedures *A* and *B* will depend largely upon the amount of acetone present; *A* gives less color than *B*, and is for larger amounts of acetone. This choice can usually be estimated roughly by inspection during the period of color development. For many purposes one procedure can be used consistently; both are described to permit the accurate covering of a wide range of acetone concentration.

For colorimetric measurement, compare the unknown in the colorimeter against a suitable standard acetone solution treated in the same way and at the same time as for the unknown.[168] For the preparation and choice of standards, see under "Calculation." For photometric measurement, determine the density in a photometer at 520 mμ, within 15 minutes after dilution by procedure *A* or 30 minutes after dilution by procedure *B*. Set the photometer to zero density with a blank prepared by treating 2 ml. of water by the same procedure as was used for the unknown.

Determination of β-Hydroxybutyric Acid. Glucose and other interfering substances are removed by the treatment with calcium hydroxide and copper sulfate as in the Van Slyke procedure, p. 930. All volumes may be reduced proportionately if advisable. If the urine is dilute, its volume may be increased in relation to the volume of the final mixture, twice the amount of copper sulfate (in 40 per cent solution) and calcium hydroxide being used. After standing for 30 to 45 minutes, the mixture is filtered, or centrifuged and

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be an excess over the alkali present, to liberate all the iodine) and titrate the excess iodine with 0.1 normal sodium thiosulfate solution, using starch as indicator. Subtract the buret reading from 50, to obtain the ml. of iodine used up. One ml. 0.1 normal iodine is equivalent to 0.9675 mg. acetone.

From the standardized acetone solution, prepare by dilution a solution containing 1 mg. of acetone per ml. This *stock standard* will keep for about 1 month. Freshly prepared *dilute standards* containing 0.1, 1.0, and 5.0 mg. per cent are made from the stock standard by diluting 0.1 ml., 1.0 ml., and 5.0 ml. respectively to 100 ml. with water and mixing. Other concentrations (for preparing a photometric calibration curve) may be made similarly.

Potassium Bichromate, 0.2 Per Cent. Dissolve 2 g. reagent-grade potassium bichromate in water and dilute to 1 liter.

[168] Behre (*loc. cit.*) describes the preparation of "artificial standards" suitable for approximate clinical purposes.

filtered. Transfer an aliquot of the filtrate (the amount depending upon the β-hydroxybutyric acid content of the urine) to a distilling flask similar to that used for the acetone determination, but in addition fitted with a dropping funnel. Make up the volume in the flask to not less than 30 ml. by adding water if necessary, and acidify with three to four drops of 1:1 sulfuric acid. Distill off one-third or more of the original volume as described for the acetone determination, to get rid of preformed acetone and acetoacetic acid. Discard the distillate; it cannot be used for simultaneous acetone determination since some acetone is lost by the treatment with calcium hydroxide and copper sulfate. Place a 200-ml. round-bottomed flask in position as receiver for the distillate, closed and containing sufficient water to cover the tip of the delivery tube as described under "Determination of Acetone," above. Bring the residual solution in the flask (volume of approximately 20 to 60 ml.) to a boil, and add through the dropping funnel 15 ml. of 1:1 sulfuric acid and 10 ml. of 0.2 per cent potassium bichromate solution, by drops, during the first five minutes of distillation, followed by 25 ml. of bichromate during each of the next two five-minute periods. Regulate the rate of distillation so that 50 to 85 ml. of distillate are obtained in 15 minutes.

Measure the volume of distillate, or dilute to a known volume with water and mix, and determine the acetone content of a 2-ml. portion as described above.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times S \times \frac{\text{Distillate Vol.}}{2} \times \frac{100}{\text{Urine Vol.}} = \begin{matrix}\text{mg. acetone per}\\ \text{100 ml. urine}\end{matrix}$$

where S is the acetone content of the standard, in mg., and the volumes of distillate, and urine whose acetone is contained in the distillate, are measured in ml. The standard should have an acetone content approximating that of the distillate. If this is not known, or will vary widely from one unknown to another, a series of five standards may be prepared which will permit the accurate estimation of any concentration of acetone in the distillate from 0.05 to 200 mg. per cent (0.001 to 4.0 mg. per 2 ml.). First prepare dilute standard acetone solutions containing 0.1, 1.0, and 5.0 mg. per cent acetone, by dilution of the stock standard. Set up five test tubes, each containing 0.1 ml. of salicylic aldehyde. Add to each the amount of a particular standard as indicated in the table, followed by 1.5 ml. of potassium hydroxide solution and subsequent treatment for color development exactly as described for the analysis of an unknown. Dilute each standard after color development to the volume and with the solvent indicated in the table. The S value for each standard, to be used in the calculation, is given in the last column of the table.

Standard	*Final Volume After Color Development, and Solvent*	*S*
1—1 ml. 0.1 mg. per cent acetone, + 1 ml. water	5 ml., alcohol	0.001
2—2 ml. 0.1 mg. per cent acetone	5 ml., alcohol	0.002
3—2 ml. 1 mg. per cent acetone	10 ml., alcohol	0.010
4—2 ml. 1 mg. per cent acetone	10 ml., water	0.020
5—2 ml. 5 mg. per cent acetone	10 ml., water	0.100

Read the unknown against the nearest standard in the same solvent. If the unknown is known to come within the range of any one standard, naturally only that standard need be prepared. If the color of the unknown in the alcohol solvent is more than twice that of Standard 3, or in the water solvent more than twice that of Standard 5, it may be diluted with the same solvent until approximate color match with a standard is obtained, and then read. Results in this event must be multiplied by the dilution. Dilution up to 200-ml. final volume is permissible.

For photometric measurement, the acetone content in the 2 ml. of distillate taken for analysis is established by reference to a calibration curve prepared previously from standard acetone solutions. The calculation is then:

$$\begin{array}{c}\text{Mg. acetone in}\\ \text{2 ml. distillate}\end{array} \times \frac{\text{Distillate Vol.}}{2} \times \frac{100}{\text{Urine Vol.}} = \begin{array}{c}\text{mg. acetone per}\\ \text{100 ml. urine}\end{array}$$

To prepare a calibration curve for procedure *A* (water solvent, 10 ml. volume), 2-ml. portions of standard acetone solutions containing from 0.0 to 0.16 mg. of acetone give a satisfactory curve relating density and concentration, at 520 mμ and 1 cm. solution depth (or its equivalent). For procedure *B* (alcohol solvent, 5 ml. volume) the corresponding range is 0.0 to 0.03 mg. of acetone. If the unknown is beyond the range of the curve, repeat the analysis using a smaller aliquot of distillate (or diluted distillate) made up to 2 ml. with water, and correct the calculations accordingly. In using a calibration curve for photometric measurement, the experimental conditions during an analysis (time of standing before and after dilution, temperature, etc.) should be uniform and should reproduce as far as possible those used when the curve was constructed. For accurate results the curve should be checked at intervals, particularly if new reagents are prepared, and reestablished if necessary.

Results obtained by the procedure in which β-hydroxybutyric acid is *not* determined (Determination of Acetone and Acetoacetic Acid) represent the preformed acetone present, as well as acetone equivalent to the acetoacetic acid present, which is decomposed into acetone during the analysis. In the β-hydroxybutyric acid analysis, results represent the *acetone equivalent* of the β-hydroxybutyric acid present. For factors used to obtain the content of β-hydroxybutyric acid itself, see previous methods.

INDICAN

Methods. Indican (indoxylsulfuric acid) is usually determined by oxidation and condensation to form indigo or similar substances, followed by extraction with chloroform or other suitable solvents, and colorimetric or photometric estimation in terms of a standard. Earlier methods employed a standard solution of indigo. This type of standard has been criticized by Meiklejohn and Cohen,[169] who state that the final color intensity obtained from urine may be deeper than that corresponding to a saturated solution of pure indigo, in the same solvent. These authors describe a photometric modification of the method of Sharlit,[170] which appears to give satisfactory results, and which is based upon calibration of the photometer with standard indican solutions. In Sharlit's procedure, developed for visual colorimetry, standard indican solutions may also be used, but the author likewise describes an artificial standard containing cobalt sulfate, whose indican equivalence is defined. Kumon[171] has described a procedure claimed to be quite specific, based upon the color reaction between indican and ninhydrin. It is not felt practical to describe any of these procedures here, because of the difficulty of obtaining pure indican for standardization purposes.[172] The reader is referred to the original papers for details.

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[169] Meiklejohn and Cohen: *J. Lab. Clin. Med.*, **27**, 949 (1941–1942). See also Townsend: *ibid.*, **23**, 809 (1937–1938).

[170] Sharlit: *J. Biol. Chem.*, **99**, 537 (1932).

[171] Kumon: *Z. physiol. Chem.*, **231**, 205 (1935).

Interpretation. The daily excretion of indican ranges from 10 to 20 mg. per day in normal individuals.

Indican is apparently formed within the body from indole produced in the intestinal lumen by bacterial action; there is no good evidence that either indole or indican are intermediates in tryptophan metabolism by animal tissues. In normal individuals, variations in indican excretion appear to be dependent mainly upon the diet, a meat diet increasing excretion while a milk or carbohydrate diet decreases excretion. Pathologically the greatest increases are found in disorders involving increased putrefaction and stagnation of intestinal contents. Bacterial decomposition of body protein, as in gangrene, putrid pus formation, etc. gives rise to increases.

PHENOLS

Method of Volterra:[173] ***Principle.*** The urine is distilled from slightly alkaline solution to obtain the free volatile phenols in the distillate. After acidification, a second distillate is obtained; this represents the conjugated volatile phenols present. Ether extraction of the remaining fluid separates the aromatic hydroxy acids from residual phenols. The significance of these various fractions is discussed under "Interpretation." Each fraction, after proper preparation, is treated with the phosphotungstic-phosphomolybdic acid color reagent of Folin and Ciocalteu and the resulting color compared with that obtained from a standard phenol solution.

Procedure.[174]

(a) FREE VOLATILE PHENOLS. **Transfer 10 ml. of the well-mixed 24-hour sample of urine to a 250-ml. distilling flask fitted with a condenser. Add 150 to 175**

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[172] For method of preparing indican from the urine of dogs fed indole, see Ellinger: *Z. physiol. Chem.*, **38**, 178 (1903).

[173] Volterra: *Am. J. Clin. Path.*, **12**, 525, 580 (1942).

[174] Reagents Required: *Silver Lactate Solution.* 3 per cent silver lactate in 3 per cent lactic acid.

Colloidal Iron. Fisher Scientific Company's "Dialyzed Iron, 5 Per Cent" is satisfactory.

Acid Sodium Chloride Solution. To 1 liter of a saturated solution of sodium chloride, add 10 ml. of concentrated hydrochloric acid.

Phenol Reagent (Folin and Ciocalteu: *J. Biol. Chem.*, **73**, 627 (1927)). Into a 1500-ml. Florence flask introduce 100 g. of sodium tungstate, $Na_2WO_4 \cdot 2H_2O$, 25 g. of sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$, 700 ml. of water, 50 ml. of 85 per cent phosphoric acid, and 100 ml. of concentrated hydrochloric acid, and reflux gently for 10 hours. Add 150 g. of lithium sulfate, 50 ml. of water, and a few drops of bromine. Boil the mixture for 15 minutes without condenser, to remove excess bromine. Cool, dilute to 1 liter, and filter. The reagent should have no greenish tint. Protect from dust. Dilute a portion with an equal volume of water before use.

Standard Phenol Solution. Prepare and standardize a stock solution of phenol, containing 1 mg. of phenol per ml., as follows: Dissolve a little over 1 g. of crystallized phenol in a liter of 0 1 N hydrochloric acid. Transfer 25 ml. of this solution to a 250-ml. flask, add 50 ml. of 0.1 N sodium hydroxide, heat to 65° C., add 25 ml. of 0.1 N iodine solution, stopper the flask, and let stand at room temperature for 30 or 40 minutes. Add 5 ml. of concentrated hydrochloric acid and titrate the excess of iodine with 0.1 N thiosulfate solution. Each ml. of 0.1 N iodine solution used up corresponds to 1.567 mg. of phenol. On the basis of the result, dilute the remainder of the phenol solution with 0.1 N acid to give a solution containing 1 mg. of phenol per ml. This stock solution is quite stable. For a ml. of water, followed by sufficient sodium bicarbonate solution to render the solution alkaline to litmus. Distil, collecting the distillate until 30 to 40 ml. have been obtained. Stop the distillation and measure the volume of distillate. Use a 10-ml. portion for the color reaction, as described below.

(b) Conjugated Volatile Phenols. When the contents of the distilling flask have cooled somewhat, add sufficient dilute sulfuric acid to render distinctly acid to Congo red, and start the distillation again. Collect 100 to 120 ml. of distillate, then test for completeness of distillation by collecting a separate 5-ml. portion of distillate and treat this by the phenol color reaction described below, using proportionately reduced amounts of reagents. If an evident color reaction is obtained in this test sample, add 50 to 100 ml. of water to the distilling flask and continue distillation until volatile phenols no longer distill over. Only in exceptional cases must more than about 150 ml. of distillate be obtained. Measure the volume of distillate, mix, and use a 10-ml. portion for the color reaction.

(c) Total Aromatic Hydroxy Acids. Transfer the acid solution in the distilling flask to a 250-ml. separatory funnel, using water for rinsing and dilution to about 100 ml., and shake for 3 minutes with 30 to 40 ml. of petroleum ether. Draw off the aqueous layer into a second separatory funnel (discard the petroleum ether layer) and shake twice with 30-ml. portions of ordinary ether, removing and combining the ether extracts, and saving the residual aqueous fluid. Wash the combined ether extracts by shaking with water, draw off the water, transfer the ether extract to an evaporating dish, and evaporate off the ether on a steam bath. Just before evaporation is complete, add about 5 ml. of water, and then complete the removal of the ether. Transfer the aqueous fluid remaining to a graduated cylinder and make up with rinsings to 10 ml. Mix, dilute 1 ml. to 10 ml. with water, and use this diluted portion for color development.

(d) Residual Phenols. Dilute the residue from the ether extraction to 200 ml. with water. To a 10-ml. portion, add 1.0 to 1.5 ml. of silver lactate solution, and one to two drops of colloidal iron. Shake, dilute to 20 ml. with water, allow to stand 15 minutes, and filter. To 10 ml. of filtrate add 1.0 to 1.5 ml. of acid sodium chloride solution, dilute to 20 ml. with water, mix, and filter. Use 10 ml. of filtrate for the color reaction.

Color Reaction. To 10 ml. of unknown in a test tube, add 0.5 ml. of diluted phenol reagent, followed by 2 ml. of 20 per cent sodium carbonate solution. Mix by shaking, and after 20 to 30 seconds place the tube in a boiling water bath for exactly 1 minute. Remove and cool in running cold water. Compare the color with that obtained by treating 10 ml. of standard phenol solution (containing 0.03 mg. of phenol) by the same procedure, at the same time.

Calculations.

Volatile Phenols (Free or Conjugated):

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{mg. phenol}}{\text{in Standard}} \times \frac{\text{ml. distillate}}{10} = \frac{\text{mg. phenol in 10 ml.}}{\text{urine}}$$

Results on the distillate from (*a*) give the free volatile phenol content and from (*b*) the conjugated volatile phenols (both expressed as phenol). From the volume of the urine, the total output per 24 hours can be calculated.

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working standard, dilute 0.3 ml. of the stock standard to 100 ml. with water and mix. This solution contains 0.03 mg. of phenol in 10 ml., and is made up fresh at the time of using.

Aromatic Hydroxy Acids:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{mg. phenol}}{\text{in Standard}} \times 10 = \frac{\text{mg. aromatic hydroxy acids}}{\text{(as phenol) in ml. urine used}}$$

Multiply results by 1.5 to express in terms of *p*-hydroxyphenylacetic acid instead of phenol. It may be necessary to dilute the 10-ml. aqueous solution of ether-soluble material, of which 1 ml. is used for analysis, to a greater volume to get color match with the standard, in which case the value 10 in the formula is replaced by the volume after dilution.

Residual Phenols:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times \frac{\text{mg. phenol}}{\text{in Standard}} \times 80 = \frac{\text{mg. residual phenols (as}}{\text{phenol) in ml. urine used}}$$

Interpretation. Results on normal and pathological urine by this method indicate that free volatile phenols (phenol, *p*-cresol, etc.) are ordinarily present only in traces, if at all, and these findings are in agreement with the results of others (Deichmann and Shafer,[175] Schmidt[176]) using different methods. Conjugated phenols range in amount from 20 to 70 mg. per day normally, and do not appear to be significantly altered in amount by diet or fasting, at least in short experiments. Pathologically, increases in conjugated phenols have been noted in conditions associated with extensive tissue destruction and in intestinal obstruction. Ingestion of phenol or benzene likewise leads to increases. Aromatic hydroxy acids (*p*-hydroxyphenylacetic acid, *p*-hydroxyphenylpropionic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenyllactic acid, and possibly *p*-hydroxyphenylpyruvic acid) are excreted normally in amounts ranging from 50 to 90 mg. (as *p*-hydroxyphenylacetic acid) per day. Pathological variations have not been extensively studied. According to Schmidt (*loc. cit.*), about two-thirds of the total aromatic hydroxy acid excretion is normally in the free form and one-third in the conjugated form. The significance of the "residual phenol" analysis is obscure. Values ranging from 150 to 500 mg. per day are found normally, but it is generally recognized that many nonphenolic substances, such as imidazoles, etc., are included in this fraction, rendering interpretation difficult. Values for residual phenols as obtained by the method described here correspond roughly to the total phenol values of the admittedly nonspecific method of Folin and Denis.[177]

SULFONAMIDES (SULFA DRUGS)

Method of Bratton and Marshall:[178] ***Principle.*** The principle is the same as that applied to the determination of sulfonamides in blood (p. 657). The protein-free urine is treated with nitrous acid to diazotize any free sulfonamide present, excess nitrous acid is destroyed, and the diazotized sulfonamide is coupled with N-(1-naphthyl)-ethylenediamine to form a stable red color which is then compared with a standard treated in the same way. Total sulfonamide is determined after hydrolysis with acid. The difference between free and total sulfonamide represents acetylated sulfonamide.

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[175] Deichmann and Shafer: *Am. J. Clin. Path.*, **12**, 129 (1942).
[176] Schmidt: *J. Biol. Chem.*, **145**, 533 (1942).
[177] Folin and Denis: *J. Biol. Chem.*, **22**, 205 (1915).
[178] Bratton and Marshall: *J. Biol. Chem.*, **128**, 537 (1939).

Procedure.[179] Transfer 1 ml. of urine to a 25-ml. volumetric flask and dilute to the mark with water. Mix, and place 2 ml. of the diluted urine in a small flask. From a buret add 30 ml. of water, followed by 8 ml. of 15 per cent tricholoroacetic acid. Mix and filter.

Free Sulfonamide. Transfer a 10-ml. portion of the trichloroacetic acid filtrate to a small flask or wide test tube, and treat with nitrite, sulfamate, etc., exactly as described for a blood filtrate (p. 658).

Total Sulfonamide. Transfer a 10-ml. portion of filtrate to a test tube or other container graduated at 10 ml., and add 0.5 ml. of 4 N hydrochloric acid. Place in a boiling water bath for 1 hour, cool, and make up to 10 ml. with water. Continue with treatment with nitrite, sulfamate, etc., as for free sulfonamide.

Compare the final color obtained against a suitable standard as described for blood analysis, the same standards being satisfactory. If the urine is unusually low or high in sulfonamide content, the determination is repeated on a more satisfactory dilution of the sample. In every case, however, the final 10-ml. portion analyzed should contain 3 per cent trichloroacetic acid. The calculation is the same as for blood analysis, except that the dilution of the urine (i.e., in the present instance the dilution is 500) replaces the value 20 in the blood calculations. Either a colorimeter or a photometer may be used, as with blood.

The procedure as described provides for urine containing protein as well as protein-free urine. If the urine is known to be free from protein, the treatment with trichloroacetic acid may be omitted. The urine is diluted so as to contain from 1 to 2 mg. per cent of sulfonamide, and then 50 ml. of this diluted urine, plus 5 ml. of 4 N hydrochloric acid are diluted to 100 ml. with water. Free sulfonamide is determined on a 10-ml. portion of this final dilution as with a blood filtrate. Total sulfonamide is determined on a 10-ml. portion, heated without further addition of acid, made up to volume, and the analysis continued as above.

Interpretation. As with blood, the sulfonamide content of urine may vary between wide limits. The procedure as described is satisfactory for urines containing from 50 to 500 mg. per cent. If smaller or larger amounts than this are present, other suitable dilutions must be made.

Sulfonamides found in the urine may be either in the form of the free drug, its acetylated derivative,[180] or oxidized forms possibly combined with glucuronic acid.[181] Only the acetylated derivative does not respond to the colorimetric procedure; the difference between free and total sulfonamide therefore represents acetylated sulfonamide. The proportion of free drug to its various derivatives depends upon a number of factors, of which the nature of the sulfonamide itself is perhaps most important; sulfanilamide is relatively little acetylated as compared to sulfadiazine, for example. Clinically, the sulfonamide content of the urine is of importance in at least two respects; these include the possible formation of urinary calculi by the insoluble and precipitated drug, and the possibility of renal damage and hematuria associated with the deposition of crystalline sulfonamide or acetylsulfonamide in the renal tubules. Studies on these two possible manifestations of sulfonamide excretion have shown that the various sulfonamides and their derivatives differ significantly in their solubility in urine and in their propensity to precipitate out, either in the renal tubules or in the urine itself; in general, the therapy consists of maintaining an alkaline urine, since the compounds responsible are more soluble at alkaline reaction. Thus far, only the free drug and its acetylated product appear to be involved here; other excretory forms of the sulfonamides, where they have been recognized, are quite soluble.

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[179] The reagents required are the same as those described on p. 658 for the determination of sulfonamides in blood.

[180] Marshall, Bratton, and Litchfield: *Science*, **88**, 597 (1938).

[181] Scudi: *Science*, **91**, 486 (1940); *also* Scudi and Jelinek: *J. Pharmacol.*, **81**, 218 (1944).

UROBILINOGEN

Method of Wallace and Diamond:[182] ***Principle.*** A series of dilutions of urine is carried to the point where the red color resulting from the reaction between urobilinogen and Ehrlich's aldehyde reagent is just discernible. This method is regarded as more accurate than the spectroscopic method of Wilbur and Addis.[183] A more extended, and probably more accurate, method for the determination of urobilinogens in urine and feces is described by Watson *et al.*[184] It has been shown that both mesobilirubinogen and stercobilirubinogen give the Ehrlich aldehyde reaction. Interfering substances can be extracted with petroleum ether.

Procedure. **One ml. of Ehrlich's aldehyde reagent[185] is added to 10 ml. of undiluted urine and allowed to stand 1 to 3 minutes. An idea as to the quantity of urobilinogen is gained by noting the rapidity and intensity of color development. Dilutions are not carried out if the color remains a light red (normal values). For higher concentrations, prepare a series of dilutions of the urine from 1:10 to 1:200, or higher, as indicated by the preliminary test. Tap water may be used, but should not be too cold. Add 1 ml. of the reagent to each dilution and after 3 to 5 minutes note the highest dilution that shows a faint pink discoloration. Express the result in terms of this dilution. The test is best performed in daylight but in the absence of bright sunlight.**

Interpretation. The appearance of the color in dilutions up to 1:20 may be regarded as normal. Constipation may produce a temporary rise in urobilinogen. This substance is believed to originate by reduction of the bile pigment. The latter may be extrahepatic as well as hepatic in origin (see p. 592). Excessive amounts of urobilinogen are, therefore, excreted in diseases of the liver and biliary tract, including toxemias of pregnancy, infectious diseases, and alcoholic intoxication, and in hemolytic diseases, including poisoning by lead, sulfonal, mushrooms, and hemolytic poisons in general.[186] It is claimed that urinary urobilinogen affords no accurate index to blood decomposition; according to Miller,

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[182] Wallace and Diamond: *Arch. Internal Med.*, **35**, 698 (1925).

[183] Wilbur and Addis: *Arch. Internal Med.*, **13**, 235 (1914).

[184] Watson, Schwartz, Sborov, and Bertie: *Am. J. Clin. Path.*, **14**, 605 (1944); Watson and Hawkinson: *ibid.*, **17**, 108 (1947).

[185] See Appendix.

[186] Sulfonamides in the urine will interfere since they likewise react with Ehrlich's aldehyde reagent. If they are present, acidify the urine and shake with petroleum ether. Shake the petroleum ether extract with aqueous alkali and carry out the test on the final neutralized aqueous extract.

Singer, and Dameshek,[187] however, the fecal output of urobilinogen is of value in this connection.

OXALIC ACID

Earlier methods for the determination of oxalic acid in urine were based on the precipitation of calcium oxalate from a large volume of urine. Difficulties in obtaining satisfactory checks were attributed to the effect of magnesium, phosphate, and sulfate ions upon the solubility and rate of crystallization of calcium oxalate. The following procedure is directed toward correcting the deficiencies in the older methods.

Total Oxalic Acid (Method of Powers and Levatin[188]): Principle. The urine is acid-hydrolyzed (to decompose any oxaluric acid) and the oxalic acid separated from interfering ions by extraction with ether. The extract is precipitated as calcium oxalate and dissolved in acid permanganate solution and back-titrated with thiosulfate.

Procedure. An all-glass continuous liquid-liquid extraction apparatus is used, as shown in Fig. 256. This consists of a 25 ml. test tube in which is placed a funnel-shaped tube made from half a 25 ml. pipet having an over-all length of about 200 mm. The stem end is sealed and 4 holes punched through the sealed end with hot wire. The tube is suspended to an outer tube (25 × 300 mm.) by means of a nichrome wire around its lip or through a small hole near the top of the inner tube. This also serves to separate the cold-finger condenser slightly thus providing a vent.

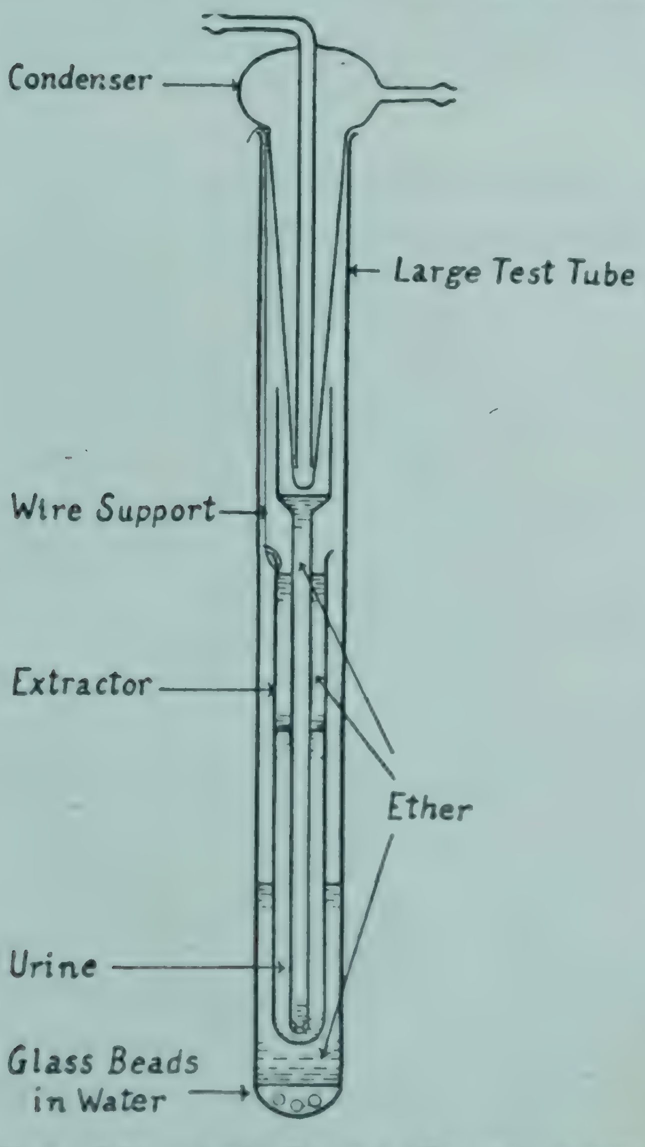

FIG. 256. ALL-GLASS CONTINUOUS LIQUID-LIQUID EXTRACTION APPARATUS.

Courtesy, Powers and Levatin.

Of the well-mixed 24-hr. urine sample (preserved if necessary with a few ml. of formaldehyde), transfer 25 ml. to a volumetric flask, add 1 ml. concentrated hydrochloric acid, mix by inversion and heat by immersion in boiling water for 30 minutes. Cool and filter.

Into the outside tube of the extraction apparatus place several glass beads, 2 ml. of water and 25 ml. of ether. Introduce 10 ml. of the acid-hydrolyzed urine filtrate into the inside tube and assemble the apparatus as shown in Fig. 256. Extraction may be carried out on an electric hot plate covered with an asbestos sheet in which a 1.5-cm. hole is cut, the walls of the extractor being thus protected from overheating and loss of ether. During the extraction a good flow of cold water must be passed through the condenser and the heating regulated so that the ether drips at the rate of about

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[187] Miller, Singer, and Dameshek: *Arch. Internal Med.*, **70**, 722 (1942).
[188] Powers and Levatin: *J. Biol. Chem.*, **154**, 207 (1944).

100 drops per minute. If necessary, add a little more ether. (*Warning*—avoid getting ether vapors near hot plate!) Extract for 6 hours.

Disconnect the apparatus. Lift the inside tube, rinsing it down with 2 ml. of ethanol from a pipet before removing. Add 1 ml. of 2 per cent acetic acid to the contents of the outside tube, shake by twirling to mix the water and ether layers (the presence of water is necessary to avoid oxalic acid loss on evaporation to dryness) and drive off the ether by immersion in a water bath at 70° C. while continuously shaking.

Transfer the aqueous residue into a 15-ml. centrifuge tube by means of an aspirating device (see Fig. 183, p. 695). The tip of the centrifuge tube should be drawn out slightly to an inside diameter of 1 mm. so as to facilitate the handling of the small precipitate. Rinse down the walls of the outside tube with two 2-ml. portions of ethanol, transferring each rinsing to the centrifuge tube.

Add 5 ml. of 10 per cent calcium chloride to the contents of the centrifuge tube and stir with a gentle current of air introduced through a fine capillary tube. Overlay with 2 ml. of acid-ethanol solution (60 ml. ethanol, 10 ml. 2 per cent acetic acid, 20 ml. water) which prevents surface accumulation of the oxalate precipitate. Let stand overnight. Centrifuge at 2,000 r.p.m. for 30 minutes, decant, and invert on filter paper to drain. Rinse down the wall of the centrifuge tube with 2 ml. of acid-ethanol solution and thoroughly break up the precipitate with a fine glass rod. Remove the rod after rinsing it with 3 ml. of acid-ethanol solution. Centrifuge, decant, and drain as above. Drive off residual alcohol by heating in an oven or boiling water bath for a few minutes.

Add 1 ml. of 20 per cent sulfuric acid and 0.5 ml. of 1 per cent manganese sulfate. Break up the precipitate with a fine glass rod which is rinsed with a few drops of water before removal. Add exactly 3 ml. 0.01 N potassium permanganate. Stir with air current and let stand 8–10 minutes. Add 0.5 ml. of 10 per cent potassium iodide, mix by twirling, and follow with 4 drops of soluble starch solution (see Appendix) and 2 drops of a saturated solution of barium hydroxide. (The barium sulfate precipitate facilitates visualization of the end point of the titration.) Titrate the excess permanganate with 0.01 N sodium thiosulfate from a 5 ml. microburet. Read to nearest 0.01 ml.

A titration reagent blank should be run in parallel. For this purpose distilled water may be used in place of the oxalate solution.

CALCULATION.

In the following equation t is the ml. of 0.01 N thiosulfate used in the back titration, b is the ml. used in the reagent blank.

$$0.45(3 - t - b) \times \frac{26}{25} \times \frac{\text{24-hour volume}}{25} = \text{mg. total oxalic acid}^{189} \text{ in 24-hour urine}$$

Interpretation. From 15 to 50 mg. of oxalic acid is excreted by a normal adult on an ordinary mixed diet. It arises from oxalates of the food ingested and from fat and protein metabolism. It is increased by the ingestion of apples, grapes, cabbage, etc., although most of the ingested oxalate is destroyed. It is increased in disturbances of metabolism associated with decreased oxidation, according to certain observers. The term "oxaluria" has been largely a misnomer.

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[189] Oxalic plus oxaluric acid. Even if preliminary acid hydrolysis were omitted, some of the oxaluric acid would be hydrolyzed at the temperature of the ether extraction.

SULFUR[190]

GRAVIMETRIC PROCEDURES

1. Total Sulfates (Folin's Method): Principle. The sulfuric acid of the conjugated sulfates is set free by boiling with acid. The total sulfates are then precipitated with barium chloride.

Procedure. **Place 25 ml. of urine in a 200- to 250-ml. Erlenmeyer flask, add 20 ml. of dilute hydrochloric acid[191] (1 volume of concentrated HCl to 4 volumes of water), and gently boil the mixture for 20 to 30 minutes. To minimize the loss of water by evaporation, the mouth of the flask should be covered with a small watch glass during the boiling process. Cool the flask for 2 to 3 minutes in running water, and dilute the contents to about 150 ml. by means of cold water. Add 10 ml. of a 5 per cent solution of barium chloride slowly, drop by drop, to the cold solution.[192] The contents of the flask should not be stirred or shaken during the addition of the barium chloride. Allow the mixture to stand at least 1 hour, then shake up the solution and filter it through a weighed Gooch crucible.[193]**

Wash the precipitate of $BaSO_4$ with about 250 ml. of cold water, dry it in an air bath or over a very low flame, then ignite,[194] cool, and weigh.

CALCULATION. Subtract the weight of the Gooch crucible from the weight of the crucible and the $BaSO_4$ precipitate to obtain the weight of the precipitate. The weight of S in the volume of urine taken may be determined by means of the following proportion:

$$\text{Mol. wt. } BaSO_4\text{:g. } BaSO_4 = \text{Mol. wt. S:}x\text{(g. S)}$$

Representing the weight of the $BaSO_4$ precipitate by y and substituting the proper molecular weights, we have the following proportion:

$$233.43:y = 32.06:x\text{(g. S in the quantity of urine used)}$$

Calculate the quantity of S in the 24-hour specimen of urine.

Interpretation. The total sulfate excretion (ethereal and inorganic sulfates) by a normal adult on a mixed diet is usually between 0.6 and

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[190] Under this heading are described gravimetric and volumetric methods for sulfur in urine. A colorimetric procedure for inorganic sulfate has been described by Kahn and Leiboff: *J. Biol. Chem.*, **80**, 623 (1928). For turbidimetric methods, see Medes and Stavers: *J. Lab. Clin. Med.*, **25**, 624 (1939–1940); Treon and Crutchfield: *Ind. Eng. Chem., Anal. Ed.*, **14**, 119 (1942).

[191] If it is desired, 50 ml. of urine and 4 ml. of concentrated acid may be used instead.

[192] A dropper or capillary funnel made from an ordinary calcium chloride tube and so constructed as to deliver 10 ml. in 2 to 3 minutes is recommended for use in adding the barium chloride.

[193] If a Gooch crucible is not available, the precipitate of $BaSO_4$ may be filtered off upon a washed filter paper (Schleicher and Schüll's, No. 589, blue ribbon), and after washing the precipitate with about 250 ml. of *cold* water the paper and precipitate may be dried in an air bath or over a low flame. The ignition may then be carried out in the usual way in the ordinary platinum or porcelain crucible. In this case correction must be made for the weight of the ash of the filter paper used.

[194] Care must be taken in the ignition of precipitates in Gooch crucibles. The flame should never be applied directly to the *perforated* bottom or to the sides of the crucible, since such manipulation is invariably attended by mechanical losses. The crucibles should always be provided with *lids* and *tight bottoms* during the ignition. In case porcelain Gooch crucibles, whose bottoms are not provided with a nonperforated cap, are used, the crucible may be placed upon the lid of an ordinary platinum crucible during ignition. The lid should be supported on a triangle, the crucible placed upon the lid, and the flame applied to the improvised bottom. Ignition should be complete in 10 minutes if no organic matter is present.

2 g. S with an average of about 1.0 g. The sulfuric acid is derived but to a slight extent ordinarily from ingested sulfates, being mainly dependent on the sulfur of the protein metabolized, and will consequently vary widely with the level of protein metabolism. From 75 to 95 per cent of the total sulfur of the urine is ordinarily found as sulfate, the proportion being greatest on a high-protein diet. The sulfate excretion is increased in all conditions associated with increased decomposition of body protein as in acute fevers and decreased whenever there is a decrease in metabolic activity.

2. *Inorganic Sulfates* (*Folin's Method*): Place 25 ml. of urine and 100 ml. of water in a 200- to 250-ml. Erlenmeyer flask and acidify the diluted urine with 10 ml. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). In case the urine is dilute, 50 ml. may be used instead of 25 ml. and the volume of water reduced proportionately. Add 10 ml. of 5 per cent barium chloride slowly, drop by drop, to the cold solution, and from this point proceed as indicated in the method under "Total Sulfates," above.

Calculate the quantity of inorganic sulfates, expressed as S, in the 24-hour urine specimen.

CALCULATION. Follow the directions given under Total Sulfates, above.

Interpretation. On an average, about 90 per cent of the total sulfates of the urine exists as inorganic sulfates, but the proportion of the sulfates existing in this form varies widely, being greater on a high protein diet than on a very low protein diet. The amount varies with the total sulfates (see above).

3. Ethereal Sulfates* (*Folin's Method*): *Principle. The inorganic sulfates are removed with barium chloride and the conjugated sulfates then determined after hydrolysis.

***Procedure.* Place 125 ml. of urine in an Erlenmeyer flask of suitable size, dilute it with 75 ml. of water, and acidify the mixture with 30 ml. of dilute hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). To the cold solution add 20 ml. of a 5 per cent solution of barium chloride, drop by drop.[195] Allow the mixture to stand about one hour, then filter it through a dry filter paper.[196] Collect 125 ml. of the filtrate and boil it gently for at least one-half hour. Cool the solution, filter off the precipitate of $BaSO_4$, wash, dry and ignite it according to the directions given on p. 946.**

CALCULATION. The weight of the $BaSO_4$ precipitate should be multiplied by 2 since only one-half (125 ml.) of the total volume (250 ml.) of fluid was precipitated by the barium chloride. The remaining calculation should be made according to directions given under "Total Sulfates," above.

Calculate the quantity of ethereal sulfates, expressed as S, in the 24-hour urine specimen.

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[195] See footnote 192, p. 946.

[196] This precipitate consists of the inorganic sulfates. If it is desired, this $BaSO_4$ precipitate may be collected in a Gooch crucible or on an ordinary quantitative filter paper and a determination of inorganic sulfates made, using the same technique as that suggested above. In this way we are enabled to determine the inorganic and ethereal sulfates in the same sample of urine.

Interpretation. The excretion of ethereal sulfates (expressed as S) varies ordinarily from 0.04 to 0.1 g. per day comprising from 5 to 15 per cent of the total sulfur excretion. The absolute amount of ethereal sulfate increases with increase in the protein of the diet and particularly with increase of putrefactive processes in the intestine or elsewhere. The amount excreted cannot, however, be taken as an index of the extent of intestinal putrefaction.

4. Total Sulfur[197] (*Benedict's Method*[198]): *Principle.* The urine is evaporated and ignited with a solution of copper nitrate and potassium chlorate. Organic matter is thus destroyed and all unoxidized sulfur is oxidized to the sulfate form and can be readily precipitated with barium chloride in the usual manner. The method is very convenient and accurate.

Procedure. Ten ml. of urine is measured into a small (7 to 8 cm.) porcelain evaporating dish and 5 ml.[199] of Benedict's sulfur reagent[200] is added. The contents of the dish are evaporated over a free flame which is regulated to keep the solution just below the boiling point, so that there can be no loss through spattering. When dryness is reached the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the bunsen burner and the contents of the dish thus heated to redness for 10 minutes after the black residue (which first fuses) has become dry. This heating is to decompose the last traces of nitrate (and chlorate). The flame is then removed and the dish allowed to cool more or less completely. Ten to 20 ml. of dilute (1:4) hydrochloric acid are then added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely 2 minutes. With the aid of a stirring rod the solution is washed into a small Erlenmeyer flask,[201] diluted with cold, distilled water to 100 to 150 ml., 10 ml. of 10 per cent barium chloride solution added drop by drop, and the solution allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighed Gooch crucible. Controls should be run on the oxidizing mixture.

CALCULATION. Follow directions given under "Total Sulfates," p. 946. Calculate the quantity of sulfur, expressed as S, in the 24-hour urine specimen.

Interpretation. The total sulfur excretion averages about 1.0 g. per day, expressed as S. It runs more or less parallel with the decomposition

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[197] For determination of total sulfur in other biological material, see the method of Stockholm and Koch (p. 643). See also Pollock and Partansky: *Ind. Eng. Chem., Anal. Ed.*, **6**, 330 (1934).

[198] Benedict: *J. Biol. Chem.*, **6**, 363 (1909).

[199] If the urine is concentrated, the quantity should be slightly increased.

[200]

Crystallized copper nitrate, sulfur-free or of known sulfur content	200 g.
Sodium or potassium chlorate	50 g.
Distilled water to	1000 ml.

Denis suggested the use of the following solution:

Copper nitrate	25 g.
Sodium chloride	25 g.
Ammonium nitrate	10 g.
Water to make	100 ml.

The procedure is the same as above except that 25 ml. of urine and 5 ml. of reagent are taken.

[201] Sometimes the porcelain glaze cracks during heating, in which case the solution should be filtered into the flask.

of endogenous and exogenous protein and a definite ratio between the excretion of total N and total S might be expected; however, no constant value can be given. See "Total Sulfates, p. 946."

5. *Total Sulfur* (*Osborne-Folin Method*[202]): *Principle.* This method depends on the destruction of organic matter by means of sodium peroxide. It is employed particularly for the determination of sulfur in foods and feces. Benedict's procedure (see above) is simpler and fully as satisfactory for urine.

VOLUMETRIC PROCEDURES

6. Volumetric Determination of Ethereal and Inorganic Sulfates and Total Sulfur (Fiske's Modification of the Method of Rosenheim and Drummond): Principle. The sulfates of the urine are precipitated by means of benzidine solution, the precipitate of benzidine sulfate being filtered off and the sulfuric acid of the compound titrated with 0.02 N NaOH, using phenolsulfonephthalein (phenol red) as an indicator. This is possible because the benzidine is a very weak base and its sulfate readily dissociates. It is necessary that excess of HCl be avoided in the precipitation process. For critical studies of this procedure, see Owen[203] and McKittrick and Schmidt.[204] Kahn and Lieboff[205] have described a colorimetric method for sulfate based on diazotization of the benzidine sulfate and coupling with phenol in alkaline solution.

Procedure.

(a) REMOVAL OF PHOSPHATE. **This step is necessary for the highest accuracy, especially where the proportion of phosphorus to sulfur may be high, as in half-hourly specimens of urine. For 24-hour specimens where the highest accuracy is not desired it may be omitted.**

Transfer to a 100-ml. volumetric flask enough urine (usually 10 to 20 ml.) to contain about 10 to 20 mg. of sulfur as sulfate. Dilute to about 50 ml. with water. Add one drop of phenolphthalein solution and then concentrated ammonium hydroxide drop by drop to a faint pink color. Add 10 ml. of 5 per cent ammonium chloride and about 1.5 g. of finely powdered magnesium carbonate. Make to mark, stopper, mix thoroughly by shaking for 1 minute, and let stand for 30 minutes. Using a dry filter, filter into a dry flask. This filtrate is used for the three following determinations.

(b) INORGANIC SULFATE. **Pipet 5 ml. of the filtrate into a large pyrex test tube with flaring lip. Add 2 drops of a 0.04 per cent alcoholic solution of bromophenol blue and 5 ml. of water. Add approximately N HCl until the last trace of blue disappears and the solution is yellow. Add 2 ml. of benzidine solution.[206] Let stand for 2 minutes. Add 4 ml. of 95 per cent acetone and let stand for 10 minutes. Prepare a thin mat of paper pulp in a filtration tube (see Fig. 257).[207] This mat should first be washed with water and then sucked dry. Filter off benzidine sulfate with very gentle suction. Wash down the** sides of the test tube with 1 ml. of 95 per cent acetone, transferring to filtration tube. Wash twice more with 1 ml. and finally with 5 ml. of acetone. Add about 2 ml. of water and poke the mat with a wire out through the bottom of the tube into the pyrex test tube, rinsing the wire with a few drops of water. Add 2 to 4 drops of 0.05 per cent water solution of phenol red. Titrate with 0.02 N NaOH (prepared from 0.1 N NaOH by dilution) the solution being kept hot. At the beginning of the titration the filter tube is kept suspended in the mouth of the test tube and the alkali run through the filter tube to dissolve adherent sulfate. Rinse the inner tube with 2 to 3 ml. of water, heat the solution to boiling so that the tube is further washed with condensed steam, and finally rinse the inner tube with a few ml. more of water and remove. Titrate to a definite pink color that remains after boiling.

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[202] Described in the eleventh and earlier editions of this book.

[203] Owen: *Biochem. J.*, **30**, 352 (1936).

[204] McKittrick and Schmidt: *Arch. Biochem.*, **6**, 411 (1945).

[205] Kahn and Lieboff: *J. Biol. Chem.*, **80**, 623 (1928).

[206] Made by shaking 4 g. of benzidine in about 150 ml. of water in a 250-ml. flask, adding 50 ml. of approximately N HCl, shaking until dissolved, and diluting to mark. Filter if necessary.

[207] Made from glass tubing of 15 mm. diameter shrunken at one end to leave an opening 3 mm. in diameter, cut to a length of 7 cm., and flanged at the cut end. A somewhat elongated tip is desirable.

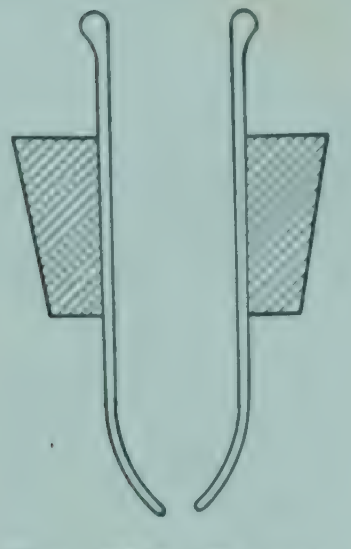

FIG. 257. FILTRATION TUBE FOR SULFUR DETERMINATION (FISKE).[196]

CALCULATION. The sulfate titrates like free sulfuric acid. One ml. 0.02 N NaOH is equivalent to 0.32 mg. S. Multiply the buret reading by 0.32 to get mg. inorganic S in 5 ml. filtrate.

(c) TOTAL SULFATE (INORGANIC AND ETHEREAL). To 5 ml. of filtrate in a 100-ml. beaker add 1 ml. of approximately 3 N HCl. Heat on a water bath to dryness and for 10 minutes longer. Transfer to a pyrex tube with lip using five 2-ml. portions of water, add 2 ml. of benzidine solution, and proceed as in the method for inorganic sulfate. The calculation is the same.

(d) ETHEREAL SULFATE. Subtract inorganic from total sulfate. The difference is ethereal sulfate.

(e) TOTAL SULFUR. Transfer approximately 0.25 ml. of Benedict's sulfur reagent (see p. 948) and 5 ml. of filtrate to a small evaporating dish (6 cm.). Evaporate carefully to dryness on wire gauze or hot plate. Gradually increase heat, finally igniting at red heat for 2 minutes over free flame. Let cool for 5 minutes. Add 1 ml. of 3 N HCl and evaporate to dryness at low heat. The mixture will turn from green to brown. Transfer to a pyrex tube with a lip with the aid of five 2-ml. portions of water. Add 1 drop of N HCl and 2 ml. of benzidine solution. Complete the determination as in the methods above but in place of the first 1-ml. portion of acetone, in washing use 2 ml. of 50 per cent acetone. The calculation is the same as above.

(f) NEUTRAL SULFUR. Subtract from the total sulfur the total sulfate as determined above. The difference is neutral or unoxidized sulfur.

Interpretation. The neutral sulfur of the urine is made up of cystine and related substances, thiocyanate, oxyproteic acids, etc. It makes up ordinarily from 5 to 25 per cent of the total sulfur of the urine, or on the average 0.08 to 0.16 g. per day calculated as S. The absolute amount is fairly constant for a given individual through wide variations of protein intake, indicating that its origin is mainly endogenous, that is, that it arises principally from the decomposition of tissue protein. On this account the percentage of the total sulfur excretion existing in the neutral form may rise to 25 per cent on a very low protein diet and decrease to 5 per cent on a high-protein diet, the absolute amount remaining nearly constant. In fasting, percentages as high as 70 have been noted. In many disorders, as tuberculosis, cancer, cystinuria, etc., the amount may be relatively and in some cases absolutely increased, but no fixed relations have been determined for the various conditions.

PHOSPHORUS

1. Determination of Inorganic Phosphate (Method of Fiske and SubbaRow[208]): Principle. Phosphate reacts with molybdic acid to form phosphomolybdic acid. On treatment with 1,2,4-aminonaphtholsulfonic acid, phosphomolybdic acid is selectively reduced[209] to produce a deep blue color ("molybdenum blue"), which is probably a mixture of lower oxides of molybdenum. This color is then compared in a colorimeter or photometer with that obtained from a suitable standard phosphate solution treated in the same way.

Procedure.[211] **Measure into a 100-ml. volumetric flask enough urine[212] to contain between 0.2 and 0.8 mg. of inorganic phosphorus (usually 1 or 2 ml.). Add water to bring the total volume to about 70 ml., followed by 10 ml. of Molybdate I reagent. Mix by gently shaking and add 4 ml. of aminonaphtholsulfonic acid reagent. Again mix, dilute to the mark with water, mix several times by inversion, and allow to stand 5 minutes.**

At the same time, transfer to a similar flask 5 ml. of standard phosphate solution, containing 0.4 mg. of phosphorus, 65 ml. of water, and the same reagents that were added to the urine sample, mixing as above. Dilute to the mark with water, mix well by inversion, and allow to stand 5 minutes. For photometric measurement, prepare a blank solution by treating 70 ml. of water in a 100-ml. flask with the same reagents that are used for the standard and unknown, dilute to the mark with water, and mix by inversion.

For colorimetric measurement, match the standard against itself at 20 mm. and compare the unknown against the standard. For photometric measurement, determine the density of the standard and of the unknown in a photometer at 660 to 720 mμ, setting the photometer to zero density with the blank.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0.4 = \begin{array}{c}\text{mg. inorganic phosphate (as P)}\\ \text{in the volume of urine used}\end{array}$$

An unknown reading between 5 and 40 mm. is acceptable with the standard at 20 mm.; if the unknown reads outside this range, repeat the analysis on a smaller or larger volume of urine. Results are usually expressed in terms of grams (or mg.) of inorganic P per 24-hour volume of urine.

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 0.4 = \begin{array}{c}\text{mg. inorganic phosphate (as P)}\\ \text{in the volume of urine used}\end{array}$$

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[208] Fiske and SubbaRow: *J. Biol. Chem.*, **66**, 375 (1925).

[209] Various other reducing agents have been proposed (see discussion on p. 630), of which stannous chloride has perhaps found most favor. A method using stannous chloride has been suggested.[210] Stannous chloride has the advantage of being more stable than aminonaphtholsulfonic acid solution, and of giving a more intense color, thus permitting the use of smaller volumes of urine. This latter appears to be of little value in urine analysis, and a disadvantage of stannous chloride is that the color produced changes in intensity on standing and may not obey Beer's law, thus requiring careful technical control, particularly in photometric measurement.

[210] Youngburg and Youngburg: *J. Lab. Clin. Med.*, **16**, 158 (1930).

[211] The reagents required, and the standard phosphate solution, are described on p. 631 in connection with the determination of blood inorganic phosphate.

[212] If albumin is present and the addition of molybdate produces a turbidity, treat the urine with 4 volumes of 10 per cent trichloroacetic acid, stopper, shake and filter, and repeat the determination using 2 to 10 ml. of filtrate. If very low in phosphate so that more than 10 ml. of filtrate must be used, follow the method for blood filtrates (see p. 631).

In a 1-cm. cuvette, and at 660 mμ, the density of the standard is approximately 0.500 (see Fig. 166, p. 632). Under these conditions, up to 0.8 mg. of phosphorus may be accurately determined. If larger amounts are present, or if a deeper cuvette is used, the determination is carried out on a smaller portion of urine (and standard, if necessary) and the calculations are corrected accordingly.

Interpretation. The excretion of phosphoric acid is extremely variable but on the average the total output for the 24 hours is about 1.1 g. expressed as P, mainly in the form of phosphates. The greater part of the phosphate excretion arises from the ingested food, either from the preformed phosphates or more especially from the organic combinations as phospho- and nucleoproteins. The excretion is consequently very largely dependent upon the phosphorus content of the diet. Some of the phosphoric acid results from the breakdown of the tissues of the body, and this endogenous phosphoric acid excretion is increased in conditions of increased metabolism as in fevers. The findings in pathological conditions have been somewhat contradictory due to lack of control of diet. The so-called "phosphaturias" nearly always represent decreased acidity and not increased phosphate content of the urine. Such conditions are, however, significant as indicating a possible tendency to the formation of phosphatic calculi.

2. Total Phosphates (Uranium Acetate Method): Principle. Standard uranium acetate is run into a measured quantity of urine until all of the phosphate has been precipitated as insoluble uranium phosphate. An excess of uranium is indicated by a reddish coloration with potassium ferrocyanide. This method is accurate and gives practically the total phosphorus of urine inasmuch as the latter exists generally almost entirely as phosphates.

Procedure. **To 50 ml. of urine in a small beaker or Erlenmeyer flask add 5 ml. of a special sodium acetate solution[213] and heat the mixture to the boiling point. From a buret, run into the hot mixture, drop by drop, a standard solution of uranium acetate[214] until a precipitate ceases to form and a drop of the mixture when removed by means of a glass rod and brought into contact with a drop of a solution of potassium ferrocyanide[215] on a porcelain test tablet produces instantaneously a brownish-red coloration. Take the buret reading and calculate the P content of the urine under examination.**

CALCULATION. Multiply the number of ml. of uranium acetate solution used by 0.002 to determine the number of grams of P in the 50 ml. of urine used. To express the result in percentage of P, multiply the value just obtained by 2, e.g., if 50 ml. of urine contained 0.074 g. P, it would be equivalent to 0.148 per cent.

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[213] The sodium acetate solution is prepared by dissolving 100 g. of sodium acetate in 800 ml. of distilled water, adding 100 ml. of 30 per cent acetic acid to the solution, and making the volume of the mixture up to 1 liter with water.

[214] *Uranium Acetate Solution.* Dissolve about 35.0 g. of uranium acetate in 1 liter of water with the aid of heat and 3 to 4 ml. of glacial acetic acid. Let stand a few days and filter. Standardize against a phosphate solution containing 0.002 g. of P per ml. For this purpose dissolve 13.480 g. of pure air-dry sodium ammonium phosphate ($NaNH_4HPO_4 + 4H_2O$) in water to make a liter. To 20 ml. of this phosphate solution in a 200-ml. beaker add 30 ml. of water and 5 ml. of sodium acetate solution (see above) and titrate with the uranium solution to the correct end-reaction as indicated in the method above. If exactly 20 ml. of uranium solution are required 1 ml. of the solution is equivalent to 0.002 g. of P. If stronger than this, dilute accordingly and check again by titration.

[215] A 10 per cent solution of potassium ferrocyanide is satisfactory. Cochineal in 30 per cent alcohol may be used as an indicator. It is added directly to the urine after the uranium acetate titration produces no further precipitate. A green color is the end reaction. The use of cochineal is more convenient but rather less accurate than the procedure involving the use of the ferrocyanide.

3. Total Phosphorus: Principle. Although urine phosphorus exists almost entirely as inorganic phosphate and the determination as such is usually sufficient, a strictly total phosphorus determination requires the destruction of organic matter. This is brought about by heating with sulfuric acid and hydrogen peroxide. The determination is otherwise the same as for the colorimetric determination of inorganic phosphate.

Procedure. **Place sufficient urine to contain between 0.2 and 0.8 mg. of total phosphorus (usually 1 to 2 ml.) in a large pyrex test tube (200 by 25 mm.), add 10 ml. of 5 N sulfuric acid (or 5 ml. of 10 N acid), and a quartz chip to prevent bumping. Heat over a microburner or on an electric hot plate until the water has been driven off and a dark brown fluid remains. Add 30 per cent hydrogen peroxide[216] by drops as necessary to complete oxidation of organic matter and leave a colorless solution, heating to boiling momentarily between drops. The hydrogen peroxide should be allowed to drop directly into the tube contents and not run down the sides of the tube. When oxidation is complete, cool slightly, add 2 to 3 ml. of water, and boil for two to three minutes to ensure hydrolysis of meta- or pyrophosphate. Cool, and transfer quantitatively with rinsings to a 100-ml. volumetric flask. Make up to about 70 ml. with water and add 10 ml. of a 2.5 per cent solution of ammonium molybdate in water. Mix and add 4 ml. of the aminonaphtholsulfonic acid reagent used for the determination of inorganic phosphate (p. 951). Dilute to 100 ml. with water and mix. The remaining procedure (including standard and calculations) is exactly as described for the determination of inorganic phosphate.**

4. Total Phosphorus (Neumann's Procedure): Principle. The organic matter is destroyed by digestion with a mixture of sulfuric and nitric acids or some other oxidizing agent. The phosphorus is then precipitated as the phosphomolybdate and determined gravimetrically or volumetrically.

Preparation of the Solution. **Pipet 10 ml. of urine (or an amount of substance containing about 10 mg. of P) into a Kjeldahl flask. Add 10 ml. of a mixture of equal parts of concentrated H_2SO_4 and concentrated HNO_3. Digest over a low flame until red fumes cease to come off. If the mixture darkens due to the charring action of the sulfuric acid, add nitric acid from a separatory funnel a few drops at a time and continue the digestion. When the mixture remains clear on evaporation to the point where white sulfuric fumes come off, the digestion is completed by heating for 10 to 15 minutes longer. Cool and transfer the solution to a 400-ml. Erlenmeyer flask with the aid of enough water to make a total volume of about 75 ml.[217]**

Instead of oxidizing the material as described above, it may be ignited with magnesia to destroy organic matter. About 2 g. of the solid substance or 25 ml. of urine (previously evaporated nearly to dryness) are mixed with a little more than an equal bulk of magnesium oxide in a porcelain dish of about 30-ml. capacity. Five ml. of magnesium nitrate solution (see Appendix) are added and the mixture heated very gently at first, then gradually to bright

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[216] Merck's Blue Label or "Special" Reagent is satisfactory.

[217] In the case of urine it is possible to neutralize this acid solution with ammonia, make it acid with acetic acid, and titrate with uranium acetate as in the preceding method.

redness. The mixture is cooled and transferred with water to a 250-ml. flask. An excess (20 to 30 ml.) of HCl are added and the mixture boiled a few minutes. Remove from the flame and add at once enough barium chloride solution to precipitate any sulfate present. Cool, make to mark, filter, and take an aliquot for analysis.

Precipitation of the Phosphomolybdate. Neutralize the solution with ammonia, make slightly acid with nitric acid, and add 15 g. of ammonium nitrate in substance (or 25 ml. of a 60 per cent solution). Heat on a water bath to 60° to 65° C. (not higher) and add 30 to 40 ml. of molybdate solution,[218] stir and let stand for about 15 minutes at 60° to 65°. Filter at once through a small paper,[219] washing the precipitate twice by decantation with 1 per cent potassium nitrate solution, using about 25 ml. each time, stirring up the precipitate well in each case, and allowing to settle. Transfer the precipitate to the filter and wash with 1 per cent potassium nitrate solution until two fillings of the filter (collected separately) do not greatly diminish the color produced with phenolphthalein by 1 drop of the standard alkali.

Titration of the Phosphomolybdate. Transfer the precipitate and filter back to the original beaker and dissolve in a small excess of 0.2 N NaOH (about 2 to 3 ml. more than required to completely dissolve the yellow precipitate). Add about 100 ml. of boiled and cooled water and a few drops of phenolphthalein as an indicator (a red color should be observed indicating excess of NaOH) and titrate the excess of NaOH with 0.1 N acid.

CALCULATION. Divide the ml. of 0.1 N acid used by 2 and subtract from the ml. of 0.2 N NaOH used. This gives the ml. of 0.2 N NaOH required. Multiply by 0.270 (the equivalent of 1 ml. 0.2 N NaOH in P) to obtain the number of mg. P in 10 ml. of the urine analyzed. Calculate the daily output of P in this case from the 24-hour volume.

Interpretation. Nearly all of the phosphorus of the urine exists as inorganic phosphates. Consequently the total phosphorus of urine varies in the same way as total phosphates. A small portion of the phosphorus of the urine may exist in organic combination, though not in a reduced form. This organically bound phosphate may amount to from 1 to 4 per cent of the total phosphorus excretion. Little is known with regard to the compounds in which it occurs. Some glycerophosphoric acid may occur either free or as lecithin.

Gravimetric Modification. The phosphorus may be determined somewhat more accurately by substituting a gravimetric procedure for the above titration. In this case the washed phosphomolybdate precipitate is dissolved on the filter paper with ammonium hydroxide and hot water to make a volume of not more than 100 ml. Nearly neutralize with HCl, cool, and add about 10 ml. of magnesia mixture (see Appendix) from a buret. Add slowly (about 1 drop per second), stirring vigorously. After 15 minutes add 12 ml.

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[218] Made by adding 5 ml. of concentrated HNO_3 to 100 ml. of the ordinary molybdate solution (see Appendix).

[219] It is better to use a special filter tube of about 1¼ inches diameter (similar to a Gooch filtering tube) in which is placed a perforated porcelain plate 1⅛ inches in diameter, covered with a layer of asbestos ⅛ inch thick. Filtration is carried out with suction and is very rapid. Ordinary Gooch crucibles lined with asbestos may also be used but are not so satisfactory. The asbestos used should be specially prepared (see Appendix). For a good discussion of the details of procedure and sources of error of this volumetric method, see Hibbard: *J. Ind. Eng. Chem.*, **5**, 998 (1913).

of ammonium hydroxide solution (sp. gr. 0.90). Let stand for some time (2 hours is usually enough), then filter and wash the precipitate with 2.5 per cent ammonia until practically free from chlorides. Ignite to whiteness or to a grayish-white ash and weigh. Multiply the weight of magnesium pyrophosphate thus obtained by 0.279 to get the weight of P.

CALCULATION. Calculate as explained above.

CHLORIDES

1. Volhard-Arnold Method: Principle. The urine is acidified with nitric acid and the chlorides precipitated with a measured excess of standard silver nitrate solution. The silver chloride formed is filtered off and in the filtrate the excess silver nitrate is titrated back with standard ammonium thiocyanate solution. Ferric ammonium sulfate is used as an indicator. A red color due to the formation of ferric thiocyanate indicates that an excess of thiocyanate is present and that the end point has been reached.

Procedure. **Place 10 ml. of urine in a 100-ml. volumetric flask, add 20 to 30 drops of nitric acid (sp. gr. 1.2) and 2 ml. of a cold saturated solution of ferric alum. If necessary, at this point a few drops of 8 per cent solution of potassium permanganate may be added to dissipate the red color. Now slowly run in a known volume of the standard silver nitrate[220] solution (20 ml. is ordinarily used) in order to precipitate the chlorine and insure the presence of an excess of silver nitrate. The mixture should be continually shaken during the addition of the standard solution. Allow the flask to stand 10 minutes, then fill it to the 100-ml. graduation with distilled water and thoroughly mix the contents. Now filter the mixture through a dry filter paper, collect 50 ml. of the filtrate, and titrate it with standardized ammonium thiocyanate solution.[221] The first permanent tinge of red-brown indicates the end point. Take the buret reading and compute the weight of sodium chloride in the 10 ml. of urine used.**

CALCULATION. The number of ml. of ammonium thiocyanate solution used indicates the excess of standard silver nitrate solution in the 50 ml. of filtrate titrated. Multiply this reading by 2, inasmuch as only one-half of the filtrate was employed, and subtract this product from the ml. silver nitrate (20 ml.) originally used, in order to obtain the ml. of silver nitrate utilized in the precipitation of the chlorides in the 10 ml. of urine employed.

To obtain the weight in g. sodium chloride in the 10 ml. of urine used, multiply the ml. of the standard silver nitrate solution actually utilized in the precipitation, by 0.010. If it is desired to express the result in percentage of sodium chloride, move the decimal point one place to the right; for results in terms of grams of sodium chloride per liter of urine, move the decimal point two places to the right.

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[220] Standard silver nitrate solution may be prepared by dissolving 29.061 g. of silver nitrate in 1 liter of distilled water. Each ml. of this solution is equivalent to 0.010 g. of sodium chloride or to 0.006 g. of chlorine.

[221] This solution is made of such a strength that 1 ml. of it is equal to 1 ml. of the standard silver nitrate solution used. To prepare the solution dissolve 13 g. of ammonium thiocyanate, NH_4SCN, in a little less than a liter of water. In a small flask place 20 ml. of the standard silver nitrate solution, 5 ml. of the ferric alum solution, and 4 ml. of nitric acid (sp. gr. 1.2), add water to make the total volume 100 ml., and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a buret until a permanent red-brown tinge is produced. This is the end-reaction and indicates that the last trace of silver nitrate has been precipitated. Take the buret reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 ml. of this solution may be exactly equal to 10 ml. of the silver nitrate solution. Make this dilution and titrate again to be certain that the solution is of the proper strength.

In a similar manner the weight or percentage of chlorine may be computed using the factor 0.006 instead of 0.010.

To express results in terms of milliequivalents of chloride per liter of urine, multiply the ml. standard silver nitrate required for the chloride in 10 ml. of urine by the factor 17.1.

Calculate the quantity of sodium chloride and chlorine in the 24-hour urine specimen.

Interpretation. From 10 to 15 g. of chlorine, expressed as sodium chloride (170 to 250 milliequivalents of chloride), are excreted per day, on the average, by normal adults. The amount is, however, closely dependent upon the chloride content of the food ingested. In fasting, the chloride excretion falls rapidly to a very minimal quantity. On high water ingestion it is increased. In pneumonia and certain other acute infectious diseases the excretion of chlorides may be markedly diminished, particularly during the periods in which exudates are forming. In convalescence and with resolution of the exudates the chlorine excretion rises again. A decrease has also been noted in nephritis associated with edema.

2. Volhard-Harvey Method: Principle. This procedure[222] differs from the Volhard-Arnold method in that the excess of silver nitrate is titrated directly without filtering and hence in the presence of the silver chloride. The procedure is thus more rapid but the end point is more difficult to determine, and the results are not so exact. It is therefore not recommended.

3. Method of Sendroy (Modified by Van Slyke and Hiller):[223] ***Principle.*** This is an application to urine of the blood-chloride method described on p. 627. The urine is shaken with solid silver iodate; chloride present forms insoluble silver chloride and an equivalent amount of soluble iodate. After removing insoluble material, the iodate in solution is converted to free iodine, which is then titrated with standard thiosulfate. The method is recommended because of its simplicity and accuracy.

Procedure.[224] **Treat 1 ml. of urine with 25 ml. of phosphoric-tungstic acid reagent and shake with silver iodate, exactly as described for the analysis of 1 ml. of plasma or serum on p. 628. After filtration or centrifugation, treat 10 ml. of the filtrate or centrifugate with sodium iodide and titrate the liberated iodine with 0.02308 N thiosulfate solution, likewise as described on p. 628.**

CALCULATION. The thiosulfate is of such strength that, at the dilution of urine employed, 1 ml. is equivalent to 10 milliequivalents of chloride per liter of urine. Therefore,

Ml. 0.02308 N thiosulfate required × 10 = milliequivalents chloride per liter urine

One milliequivalent of chloride equals 0.0585 g. of sodium chloride.

If the chloride content of the urine is so low that less than 5 ml. of thiosulfate are required for the titration, repeat the analysis, using 5 ml. of urine and 25 ml. of phosphoric-tungstic acid reagent. Shake with iodate and titrate a 10-ml. portion of filtrate as before, and calculate as follows:

Ml. 0.02308 N thiosulfate required × 2.31 = milliequivalents chloride per liter urine

Multiply the result by 0.0585 to express in terms of g. sodium chloride per liter.

Interpretation. See above.

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[222] Harvey: *Arch. Internal Med.*, 6, 12 (1910).

[223] See footnote 209, p. 627.

[224] Reagents Required: See method for blood chlorides, p. 627.

FLUORINE

Determination of Fluoride (Method of Icken and Blank):[225] ***Principle.*** Fluoride ions react quantitatively to form a complex with thorium alizarin lake, resulting in a bleaching of the color. The residual intensity is read spectrophotometrically and interpolated on a reference curve derived from standard sodium fluoride solutions. The determination of fluorides in urine is preceded by the separation of phosphates and sulfates (by distillation from perchloric acid[226]) and of chlorides (by precipitation with silver sulfate[227]).

Procedure. (a) ISOLATION OF FLUORIDES.[228] **Evaporate to dryness an aliquot of a 24-hour specimen of urine, containing from 100 to 200 μg. fluorine (usually about 200 ml.), by adding it in small portions to 25 ml. of a suspension of calcium hydroxide[229] in a platinum dish or crucible. Dry thoroughly under an infrared lamp, then char slowly and finally ignite in a muffle oven at 600° C. until all traces of organic matter disappear.**

Cool and wet the ash with 10 ml. distilled water. Dissolve the ash in a minimum quantity of perchloric acid,[230] keeping the dish covered with a watch glass. Rinse down the cover with a little water and transfer the solution through a funnel to a suitable distillation apparatus, e.g., a constant-temperature still.[231] Rinse the dish and the funnel with perchloric acid, using 20 ml. for all the above mentioned operations. Add to the mixture in the still a quantity of crystalline silver sulfate equivalent to the content of chlorides, previously determined on a separate aliquot of the urine by one of the methods described under "Chlorides." Connect the still to a steam generator and steam-distil, collecting about 200 ml. of distillate.

Evaporate to dryness and ignite the distillate as previously described, using an amount of the calcium hydroxide suspension sufficient to assure alkalinity. Dissolve the ash in perchloric acid and transfer to the still, which has been washed with hot 10 per cent NaOH and thoroughly rinsed with distilled water. Add to the solution in the still 200–500 mg. crystalline silver sulfate to precipitate the remaining traces of chlorides. Repeat the steam distillation and collect the distillate in a 150 ml. volumetric flask to which a few drops of *p*-nitrophenol indicator[232] and a few drops of 0.05 N KOH have been added. Keep the distillate alkaline at all times by dropwise addition of 0.05 N KOH. So regulate this addition of alkali that the distillate is neutralized as it approaches the mark.

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[225] Icken and Blank: *Anal. Chem.*, **25**, 1741 (1953).

[226] Willard and Winter: *Ind. Eng. Chem., Anal. Ed.*, **5**, 7 (1936).

[227] McClure: *Ind. Eng. Chem., Anal. Ed.*, **11**, 171 (1939).

[228] Association of Official Agricultural Chemists: *Official Methods of Analysis* (1950), with minor modifications.

[229] *Calcium hydroxide suspension.* Carefully slake *ca.* 56 g. of low fluorine CaO with 250 ml. distilled water, and add slowly and with stirring 250 ml. of 60 per cent $HClO_4$. Add a few glass beads and boil down to copious fumes of acid. Cool, add 200 ml. of H_2O and boil down again. Repeat diluting and boiling down once more. Cool, dilute, and filter through fritted glass filter if precipitate of SiO_2 has appeared. Pour solution, with stirring, into 1 l. of 10 per cent NaOH solution, allow precipitate to settle. Siphon off the supernatant liquid. Remove sodium salts by washing and centrifuging 5 times. Finally shake precipitate into suspension and make to 2 liters. Preserve in paraffined bottles. Shake suspension well before using.

[230] *Perchloric acid.* Dilute 60 per cent $HClO_4$ with 3 volumes of distilled water and boil down to original volume. Avoid active fuming. Repeat and preserve in pyrex glass bottle.

[231] Huckabay, Welch, and Metler: *Anal. Chem.*, **19**, 154 (1947).

[232] *p-Nitrophenol indicator:* 0.5 per cent alcoholic solution.

(b) DETERMINATION OF FLUORIDES. **Pipet standard solutions of sodium fluoride[233] containing 0, 10, 20, 30, 40, 50 and 500 μg. of fluoride ion, and an aliquot of the final distillate containing from 0 to 50 μg. of fluoride ion, into 50 ml. volumetric flasks. Add 10 ml. of the thorium-alizarin reagent[234] to each flask. Make the flasks to volume with distilled water, stopper, shake, and allow to stand for 2 hours. Determine the light absorbance of the standards at a wavelength of 525 mμ by means of a spectrophotometer equipped with 10-mm. Corex cells. Set the instrument for 100 per cent transmittance with the 500 μg. standard. Plot the reference curve. Determine the light absorbance of the sample and read the fluoride content from the reference curve.**

CALCULATION.

a = ml. of urine taken for analysis

b = ml. of final distillate taken for the spectrophotometric determination

c = μg. fluoride in aliquot "b" (from reference curve)

$$\frac{c \times 150}{a \times b} = \text{mg. F}^- \text{ per liter urine}$$

Interpretation. The amount of fluorine in urine is influenced by the fluoride content of the diet or water supply and is therefore a criterion of fluorine exposure and absorption. In areas where dental fluorosis (mottled enamel) is prevalent an average concentration of 3 mg. F per liter of urine has been reported as compared with a "normal" value of 1 mg. per liter.[235] Two populations whose water supplies contained 0.06 and 1.36 parts per million (mg. per liter) of fluoride respectively, (the latter by fluoridation) showed mean urine fluoride levels of 0.06 ± 0.13 and 1.12 ± 0.56 mg. F per liter and blood levels of 0.4 and 4.0 μg. per 100 ml., respectively.[236] Fluoride is eliminated not only via urine and feces, but also by insensible and sensible perspiration. No significant retention was noted when the daily intake was below 5 mg., the data suggesting this to be the approximate limit.[237]

TOTAL FIXED BASE

Method of Fiske:*[238] *Principle. The fixed bases sodium, potassium, calcium, and magnesium are converted into sulfates and the combined sulfuric acid titrated by the benzidine method.

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[233] *Sodium fluoride standard solution:* The sodium fluoride content of a commercial sodium fluoride c.p. powder is determined by precipitation as CaF_2 (cf. Scott, W. W., *Standard Methods of Chemical Analysis*, Vol. I, 4th ed., p. 226, 1925). 2.2105 g. sodium fluoride (100 per cent basis) are dissolved in distilled water in a 1-l. volumetric flask and made to volume. (0.5 ml. = 500 μg. F^-.) Ten ml. of this solution are diluted to 1 l. with distilled water to give the final standard solution (1 ml. = 10 μg. F^-).

[234] *Thorium-alizarin reagent:*

10 ml. 0.1 N hydrochloric acid.
10 ml. 1 per cent hydroxylamine hydrochloride.
40 ml. 0.1 per cent aqueous alizarin sodium monosulfonate.
40 ml. 0.05 per cent thorium nitrate, $Th(NO_3)_4{\cdot}4H_2O$.

The components are added in the sequence listed. The reagent is prepared fresh from stock solutions.

[235] Greenwood: *Physiol. Revs.*, **20**, 582 (1940).

[236] Smith, Gardner, and Hodge: *J. Dent. Res.*, **29**, 596 (1950).

[237] McClure, Mitchell, Hamilton, and Kinsner: *J. Ind. Hyg. Toxicol.*, **27**, 159 (1945).

[238] Fiske: *J. Biol. Chem.*, **51**, 55 (1922). See also method of Stadie and Ross: *J. Biol. Chem.*, **65**, 735 (1925) and comment of Brown and Shohl: *J. Biol. Chem.*, **91**, 745 (1931). A simple and accurate method for the determination of total fixed base by electrolysis is described by Consolazio and Talbot: *J. Biol. Chem.*, **132**, 753 (1940).

Procedure. **Measure into a large-lipped test tube (200 by 20 mm.) a sample of urine containing preferably between 10 and 25 mg. of NaCl but not more than 5 mg. of inorganic phosphorus. Add 1 ml. of approximately 4 N sulfuric acid and 0.5 ml. of concentrated nitric acid, and boil down until white fumes appear. If the residue does not soon become colorless after this stage has been reached, cool slightly, add a few more drops of nitric acid, and continue the heating. When the remaining drop of sulfuric acid has become clear and colorless, let cool for a few minutes, and wash into a test tube which is marked at 25 ml., with four 2-ml. portions of water. Add a drop of saturated alcoholic solution of methyl red. Neutralize with powdered ammonium carbonate until the color of the indicator just begins to change, and restore the pink color by adding 4 N sulfuric acid 1 drop at a time. Heat to boiling and add more acid to restore the pink color if this is necessary. Add a 10.5 per cent solution of ferric chloride crystals in 0.2 N HCl in the proportion of 0.1 ml. for each mg. of inorganic phosphorus present, shake and run in 1 ml. of a 5 per cent solution of ammonium acetate. Add sufficient water to make the total volume 10 or 11 ml., heat again to boiling, and dilute to the 25-ml. mark with cold water. Insert a rubber stopper and mix by inverting a few times. Filter at once through a dry 9-cm. ashless paper into a dry test tube. Keep the filter nearly filled with liquid as long as possible and collect only 20 ml. Stopper the tube containing filtrate and cool. The phosphate has now been removed.**

Transfer 5 ml. of filtrate to a small platinum dish, add 1 ml. of approximately 4 N sulfuric acid, and evaporate on the water bath until nearly dry. Place the dish on a metal triangle and heat cautiously at first over a microburner, gradually raising the flame until fumes have ceased to come off. Let cool, sprinkle over the residue a little powdered ammonium carbonate, and ignite again, finally raising the flame to its maximum and moving the triangle about until each part of the dish has been momentarily subjected to a dull red heat. When the dish has cooled, add 2 ml. of water. Agitate until the residue has dissolved, using a rubber-tipped rod to assist in solution if necessary. Transfer the contents of the dish to a large-lipped pyrex test tube, rinsing four times with 2-ml. portions of water. Determine the sulfate according to the benzidine method (see p. 949).

CALCULATION. An aliquot of one-fifth of the original urine was used for the determination and 0.02 N NaOH in the titration. Therefore the titration reading is equivalent to the number of ml. of 0.1 N fixed base in the sample of urine used. Subtract a correction of 1 per cent for the contraction of the warm solution during the filtration stage. Results are usually expressed in terms of milliequivalents of total fixed base per liter: this is obtained by dividing by 10 the number of ml. of 0.1 N base per liter.

Interpretation. The total fixed base excretion, combining as it does the sodium, potassium, calcium, and magnesium excretions, will be influenced by factors affecting any of these. In acidosis, volatile base (ammonia) plays a large part in neutralization but the fixed base excretion is also increased in varying degrees.

CALCIUM

Determination of Calcium* (*Method of Shohl and Pedley*[239]): *Principle. The urine is oxidized with ammonium persulfate. Calcium is precipitated as oxalate and titrated with potassium permanganate. The method is more rapid than the gravimetric.

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[239] Shohl and Pedley: *J. Biol. Chem.*, **50**, 537 (1922). For references to various other procedures upon which the determination of calcium may be based, see the section on blood-calcium methods in Chapter 23, p. 644.

***Procedure.* To 100 ml. of unfiltered urine in a 250-ml. Erlenmeyer flask add 5 ml. of concentrated HNO_3 or H_2SO_4 and one spoonful (containing 3 to 4 g.) of ammonium persulfate. Insert a funnel in the flask to prevent spattering. Boil and keep near the boiling point on a hot plate or over a low flame for 1 hour or until reduction of the persulfate is complete, as evidenced by an absence of frothing when the flask is agitated. The solution at this point is pale green in color. Add 10 ml. of 2.5 per cent oxalic acid. Cool to room temperature. Neutralize with ammonium hydroxide, using one drop of methyl red as an indicator. Cool to room temperature. If the color is now red, add a few drops of ammonia to bring to intermediate color between red and yellow (pH 4.8 to 5.2). Let stand overnight. Filter. Whatman No. 50 hardened filter paper 12.5 cm. is satisfactory. Wash precipitate and flask three times with distilled water,[240] filling the filter two-thirds full each time and allowing to drain. Break a hole in the filter paper and wash back the precipitate into the original flask, first with distilled water and then with hot dilute sulfuric acid, bringing the volume to about 100 ml. Add 10 ml. of concentrated sulfuric acid and heat to 70° to 80° C. Titrate with 0.05 N potassium permanganate, taking as an end point the first color that persists 15 to 30 seconds. One ml. 0.05 N $KMnO_4$ is equivalent to 0.0010 g. Ca.**

Interpretation. The average urinary excretion of calcium by normal adults lies between 0.1 to 0.3 g. (expressed as Ca) per day. This corresponds to 5 to 15 milliequivalents of calcium ion. Calcium excretion in the urine is dependent very largely upon the amount of calcium in the diet. From 10 to 40 per cent of the ingested calcium ordinarily is excreted by this channel, the greater part being eliminated by the feces. The proportion is dependent particularly on the amount of calcium in the food. If the calcium ingestion is very high, the per cent of the total excretion taking place by way of the kidneys will be low, and *vice versa*. As excretion takes place by way of the intestine as well as by the kidneys, no conclusions can be drawn from urinary analyses alone. The excretion of calcium may be greatly increased in certain bone disorders as osteomalacia. In others, as in rickets, the urinary excretion may be very low. For further discussion, see p. 820.

The calcium content of the urine is of clinical significance in connection with the formation of certain calcium-containing stones. According to Shorr,[241] measures designed to decrease the amount of calcium (and of phosphate) in the urine, or to increase the solubility of calcium as by promoting the urinary excretion of citrate (which forms a soluble complex with calcium) should prove to be of value in the management of nephrolithiasis due to stones of the calcium-carbonate and calcium-phosphate types.

CALCIUM AND MAGNESIUM

McCrudden's Methods: Principle. Urine contains magnesium, phosphates, and a small amount of iron, each of which will interfere with the accurate determination of its calcium content if proper conditions of acidity are not maintained during the precipitation. In the following method the proper acidity is attained through the use of sodium acetate and hydrochloric acid, and this with slow addition of the ammonium oxalate reduces the danger of occlusion of magnesium oxalate, calcium phosphate, or ferric phosphate in the calcium oxalate precipitate.

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[240] See footnote 249, p. 645.

[241] Shorr: *J. Urol.*, 53, 507 (1945).

The calcium oxalate precipitate is either ignited and weighed as CaO or determined volumetrically by titration with potassium permanganate. Magnesium is determined in the filtrate from the calcium determination after destruction of the organic matter. It is determined in the usual way by ignition of the magnesium ammonium phosphate precipitate and weighing as the pyrophosphate.

For colorimetric adaptations of these classical methods for calcium or magnesium, reference should be made to the procedures described for blood in Chapter 23.

Calcium: Gravimetric Procedure. **If the urine is alkaline, make it neutral or slightly acid and filter. Take 200 ml. of the filtered urine for analysis. If it is only faintly acid to litmus paper, add 10 drops of concentrated hydrochloric acid (sp. gr. 1.20). If the urine is strongly acid, it may be made just alkaline with ammonia and then just acid with hydrochloric acid, after which the 10 drops of concentrated hydrochloric acid are added. Then add 10 ml. of 2.5 per cent oxalic acid. Run in slowly with stirring 8 ml. of 20 per cent sodium acetate. Allow to stand overnight at room temperature or shake vigorously for 10 minutes. Filter off the precipitate of calcium oxalate on a small paper, and wash free from chlorides with 0.5 per cent ammonium oxalate solution. The precipitate may then be dried, ignited to constant weight, and weighed as calcium oxide, or it may be manipulated volumetrically as described below.**

Volumetric Procedure. **If free from uric acid, the calcium oxalate precipitate may be washed three times with distilled water, filling the filter about two-thirds full and allowing it to drain completely before adding more. A hole is made in the paper and the calcium oxalate washed into a clean flask. The volume of the fluid is brought up to about 50 ml. and 10 ml. of concentrated sulfuric acid added. Titrate with standard potassium permanganate solution to a pink color which endures for at least a minute.**

CALCULATION. In the gravimetric procedure, convert the weight of CaO into terms of Ca by multiplying by 0.715. In the volumetric procedure, one ml. of 0.1 N permanganate solution is equivalent to 2.0 mg. of Ca. Calculate the daily output of calcium.

Magnesium: Gravimetric Procedure. **Transfer the filtrate from the determination of calcium as above to a porcelain dish, add about 20 ml. of concentrated nitric acid, and evaporate to dryness. Heat the residue over a free flame until the ammonium salts are destroyed and the residue fuses. After cooling, take the residue up with water and a little hydrochloric acid and filter if necessary. Dilute to about 80 ml., nearly neutralize with ammonia, and cool. Add a slight excess of sodium acid phosphate and then ammonia drop by drop with constant stirring until the solution is alkaline, and then add enough more slowly with constant stirring to make the solution contain one-fourth its bulk of dilute ammonia (sp. gr. 0.96). Allow to stand overnight. Filter and wash free from chlorides with alcoholic ammonia solution (1 part alcohol, 1 part dilute ammonia, 3 parts water). The precipitate with filter paper is incinerated slowly and carefully with a good supply of air to prevent reduction, in the usual manner, and ignited and weighed as the pyrophosphate.**

CALCULATION. To obtain the weight of Mg, multiply the weight of magnesium pyrophosphate by 0.2184.

Interpretation. The daily excretion of magnesium by way of the urine usually amounts to between 0.05 and 0.2 g. (expressed as Mg). This amount corresponds to 4 to 20 milliequivalents of magnesium ion per day. The amount depends mainly upon the diet. Usually less than 50 per cent of the excreted magnesium is eliminated by the kidneys, the major portion passing out in the feces. The proportion varies, however, and it is impossible to draw any conclusions from the urinary output alone. There may be a retention of magnesium in certain bone disorders accompanying a loss of calcium—in osteomalacia, for example. Thus the excretions of calcium and magnesium do not necessarily run parallel.

Determination of Calcium in Ash of Foods or Feces.[242] **Ignite the material in a crucible to a white ash and dissolve the ash with the aid of a little hydrochloric acid. Bring the volume of the ash solution to 75 to 150 ml. Make just alkaline with strong ammonia added drop by drop (using litmus paper or alizarin as an indicator). Add concentrated HCl drop by drop until just acid to litmus. Then add 10 drops of concentrated HCl (sp. gr. 1.20) and 10 ml. of 2.5 per cent oxalic acid. Either of two procedures may then be followed. (*a*) The solution is boiled until the precipitated calcium oxalate is coarsely crystalline, and then an excess of 3 per cent ammonium oxalate is slowly added to the boiling solution and the boiling continued until any further precipitate is coarsely crystalline. (If but little calcium is present, nothing will precipitate at this point and it is not necessary to add oxalate.) Or (*b*) the flask closed with a rubber stopper is shaken vigorously for 10 minutes. An excess of 3 per cent ammonium oxalate is then added. Cool to room temperature. Add 8 ml. of 20 per cent sodium acetate solution. (In case of ash of feces add 15 ml.) The solution may either be (*a*) allowed to stand overnight or (*b*) stoppered and vigorously shaken for 10 minutes. The calcium oxalate is filtered off on a small ash-free paper and washed free from chlorides with 0.5 per cent ammonium oxalate solution. Either of two procedures may next be followed: (*a*) The precipitate and filter are dried, and burned in a platinum or porcelain crucible to constant weight as CaO. (*b*) The precipitate is washed three times with cold distilled water, as given under the method for urine and the oxalate titrated with potassium permanganate.**

Magnesium is determined in the filtrate from calcium just as given above.

SODIUM AND POTASSIUM

Determination of Combined Sodium and Potassium.[243] **From 50 to 100 ml. of urine, depending upon the specific gravity, are oxidized in a Kjeldahl flask with nitric and sulfuric acids as in the Neumann procedure for total phosphorus (see p. 953). To remove the sulfuric acid as completely as possible, transfer with the aid of a little water to a platinum dish[244] and evaporate to dryness over a free flame. (The alkalies are in the form of sulfate and do not**

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[242] For another procedure for the determination of calcium in tissues and other biological materials, see p. 648 (Corley and Denis: *J. Biol. Chem.*, **66**, 601 (1925)). For determination of Ca, Mg, and P in materials such as hay and cow feces which contain much acid-insoluble material, see Morris, Nelson, and Palmer: *Ind. Eng. Chem., Anal. Ed.*, **3**, 164 (1931).

[243] For a method for direct quantitative determination of sodium, potassium, calcium, and magnesium in urine and feces, see Tisdall and Kramer: *J. Biol. Chem.*, **48**, 1 (1921). The flame photometer (see p. 530) of Barnes, Richardson, Berry, and Hood (*Ind. Eng. Chem., Anal. Ed.*, **17**, 605 (1945)) is being successfully used in many laboratories for the simple and accurate separate determination of urinary sodium and potassium.

[244] Wilson (*J. Biol. Chem.*, **50**, 301 (1922)) suggests the use of inexpensive tin dishes instead of platinum.

volatilize.) Dissolve the residue in hot water with the aid of a little dilute hydrochloric acid. Heat to boiling and add barium chloride solution until no more precipitate forms. While still hot, add an excess of ammonia and ammonium carbonate. The barium chloride precipitates the sulfates and part of the phosphates: the ammonia in the presence of excess barium precipitates the rest of the phosphates, and the carbonate precipitates the calcium and most of the magnesium, as well as the excess barium. Filter and wash the precipitate well with hot water containing a few drops of ammonia. Evaporate the filtrate and washings to dryness and heat the residue to dull redness for a moment. Redissolve in water and treat again with ammonia and ammonium carbonate to remove any remaining alkaline earth metals. Filter and wash as before. Transfer the filtrate and washings to a weighed platinum dish, add a few drops of hydrochloric acid, and evaporate to dryness. Heat the residue gently to remove ammonium salts and then to dull redness for a moment. Desiccate and weigh. Reheat to constant weight which represents the combined chlorides of sodium and potassium. The reagents used in the determination must be tested and found free from alkali metals or a correction made for the alkali metals present in the reagents used. The sodium is determined by difference after potassium has been estimated by the method given below.

Potassium. **Dissolve the alkali chlorides from the preceding determination in a little water and add a slight excess of 10 per cent platinic chloride over that necessary to precipitate all of the alkali present calculated as sodium chloride (about 17 ml. being required for each gram of sodium chloride). Evaporate to a syrupy consistency on the water bath and add about 50 ml. of 80 per cent alcohol. Stir occasionally for a few minutes. This operation must be carried out in the absence of ammonia vapors. Filter through a weighed Gooch crucible, washing the precipitate with 80 per cent alcohol first thoroughly by decantation and then on the filter, for some time after the filtrate is colorless. Dry at 110° to 115° C. and weigh.**

CALCULATION. Multiply the weight of potassium platinic chloride by 0.1608 to obtain the amount of K present. Express as KCl by using instead of this factor the factor 0.3067. Subtract from the weight of total alkali chlorides as determined in the preceding method, the weight of potassium chloride as calculated and obtain the amount of sodium chloride present. To convert sodium chloride into sodium, multiply by 0.3934. To express the sodium (or potassium) content in terms of milliequivalents, divide the weight of NaCl (or KCl) in grams by 0.05845 (or 0.07455).

Interpretation. The average alkali excretion of an adult on a mixed diet is about 1 to 3 g. of potassium expressed as K (40 to 65 milliequivalents), and 3.0 to 5 g. of sodium expressed as Na (130 to 200 milliequivalents). The ratio of Na to K is thus about 5:3. Both the ratio and the absolute amounts of these elements excreted are, however, largely dependent upon the salt content of the diet. The urine during fasting contains more potassium than sodium salts, because of the noningestion of sodium chloride and the accompanying destruction of potassium-containing body tissues. The excretion of the bases, particularly K, may be increased in fevers and in acidosis.

IRON

Methods of Elvehjem and Kennedy. The urine is ashed, the ash dissolved, and the iron present determined colorimetrically as thiocyanate.

Procedure. **Evaporate and ash 100 ml. of urine and carry out iron determination according to Elvehjem or Kennedy (see p. 656).**

IODINE

Method of Von Fellenberg:[245] ***Principle.*** For discussion of the principle, see the original articles. McCullagh[246] has suggested a simpler procedure for iodine determinations in blood and other materials. Chaney's method[247] for protein-bound iodine is described on p. 661.

Procedure. **To 10 to 40 ml. of urine add 1 to 3 ml. of a saturated solution of iodine-poor K_2CO_3 and evaporate in a low iron dish (about 10 cm. diameter). Heat gently without igniting, moisten with water, and ignite, not bringing the bottom of the dish to redness. Extract the charred mass with a little water and filter. Ignite the paper and residue, then add the filtrate (only faintly yellow) and a few drops of 10 per cent $NaNO_3$, and complete ignition. The residue should be pure white. Extract four times with 2 to 3 ml. of alcohol. Evaporate the alcoholic solution in a gold or platinum dish (about 6 cm. diameter) on a water bath to dryness, after adding a few drops of saturated K_2CO_3 solution. Ignite gently. Extract again with 95 per cent alcohol and evaporate and ignite, this time adding no K_2CO_3. The bottom of the dish must not turn red. Dissolve the residue in 0.3 ml. of water and titrate iodine according to the method of McCullagh[246] or according to the procedure of v. Fellenberg.[248] The amount of iodine eliminated in 24 hours may be from 10 to 200 μg.**

REFERENCES TO OTHER QUANTITATIVE METHODS FOR URINE

Arsenic. Lespagnol, Merville, and Werquin: *Bull. soc. chim. biol.*, **25**, 322 (1943).
Choline. Luecke and Pearson: *J. Biol. Chem.*, **153**, 259 (1944).
Citric Acid. Goldberg and Bernheim: *J. Biol. Chem.*, **156**, 33 (1944).
Corticosteroids. Romanoff, Plager, and Pincus: *Endocrinology*, **45**, 10 (1949); Pincus and Romanoff: *Fed. Proc.*, **9**, 101 (1950); Engel: *Recent Progress in Hormone Research*, **6**, 277 (1951).
Dihydroxyacetone. McClellan: *J. Biol. Chem.*, **76**, 481 (1928).
Ethyl Alcohol. Friedemann: *J. Biol. Chem.*, **123**, 161 (1938); Kozelka and Hine: *Ind. Eng. Chem., Anal. Ed.*, **13**, 905 (1941).
Guanidine Bases. Weber: *J. Biol. Chem.*, **78**, 465 (1928); Andes and Myers: *J. Biol. Chem.*, **118**, 137 (1937).
Homogentisic Acid. Briggs: *J. Biol. Chem.*, **51**, 453 (1922).
Inulin. Ranney and McCune: *J. Biol. Chem.*, **150**, 311 (1943).
Keto Acids. Friedemann and Haugen: *J. Biol. Chem.*, **147**, 415 (1943).
Lead. Clifford and Wichmann: *J. Assoc. Official Agr. Chem.*, **19**, 130 (1936); Horowitz and Cowgill: *J. Biol. Chem.*, **119**, 553 (1937); Kaye: *J. Lab. Clin. Med.*, **28**, 1171 (1942–1943).
Mercury. Van Zwet and Duran: *Chem. Weekblad*, **38**, 186 (1941).
Zinc. Cholak, Hubbard, and Burkey: *Ind. Eng. Chem., Anal. Ed.*, **15**, 754 (1943).

For the determination of urinary 17-ketosteroids, see Chapter 26. For the determination of various vitamins in urine, see Chapter 35. See also the references to methods for blood analysis (Chapter 23) since many of these are equally suitable for urine.

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[245] The method given is a modification by Lunde: *Biochem. Z.*, **193**, 94 (1928).
[246] McCullagh: *J. Biol. Chem.*, **107**, 35 (1934).
[247] Chaney: *Anal. Chem.*, **22**, 939 (1950).
[248] von Fellenberg: *Ergeb. Physiol.*, **25**, 176 (1926); *Biochem. Z.*, **224**, 170 (1930); Lunde, Closs, and Pedersen: *Biochem. Z.*, **206**, 261 (1929).

TESTS FOR KIDNEY EFFICIENCY

1. Blood-Urea Clearance** (**Method of Möller, McIntosh, and Van Slyke**):*[249] ***Principle. Urea is normally filtered through the renal glomeruli and only partly reabsorbed in the tubules. The blood-urea clearance test is a measure of the efficiency with which the kidney excretes urea. Results are expressed either in terms of the volume of blood in ml. which is completely cleared of urea per minute, or more commonly as the percentage of the average normal value which this volume represents. The necessary data include the concentration of urea in blood and urine and the volume of urine excreted in unit time.

***Procedure.* The patient is permitted to eat a moderate meal (usually breakfast) without coffee. Vigorous exercise is avoided and after breakfast the patient is kept at rest during the 2-hour test period. At the beginning of the test period, the bladder is emptied and the urine discarded. The patient is given a glass of water to drink, and the urine is collected in two succeeding periods of one hour each. At the beginning of the second period, the patient is given another glass of water, and a sample of blood is drawn for urea determination. At the close of the test, the volumes of urine passed during each period are accurately measured in graduated cylinders, and the urea content of each determined separately.**

The two collection periods need not be exactly one hour long, but may be longer if convenient. It is important, however, that the *time* of the period be accurately known, to the nearest minute, and that the entire volume of urine formed during this period be obtained, since only in this way can the volume of urine flow per minute be calculated. The two periods serve as checks on one another; if results calculated on the basis of each separate period differ significantly, error is indicated. If the urine volume is less than 25 ml. per hour, the sample is discarded.

CALCULATION. The *maximum clearance* (C_m) indicates the maximum efficiency of urea excretion with high urine volumes and is calculated if the urine volume observed in an adult, or if the corrected volume $V \times \dfrac{1.73}{\text{sq. m. surface area}}$ in a child, exceeds 2 ml. per minute. The *standard clearance* (C_s), or the efficiency with which the kidneys excrete urea when the urine volume is at the average normal level of 1 ml. per minute, is calculated if the urine volume, corrected in the case of a child, is less than 2 ml. per minute. Both clearances are best calculated in percentage of the average normal C_s or C_m.

$$\text{Percentage of average normal } C_m = \frac{100\,UV}{75B} = 1.33\,\frac{UV}{B}$$

$$\text{Percentage of average normal } C_s = \frac{100\,U\sqrt{V}}{54B} = 1.85\,\frac{U\sqrt{V}}{B}$$

U = Urea concentration of urine (mg. urea N per 100 ml.)
B = Urea concentration of blood (mg. urea N per 100 ml.)
V = ml. urine excreted per minute

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[249] Möller, McIntosh, and Van Slyke: *J. Clin. Invest.*, **6**, 427 (1928). See also Van Slyke and Cope: *Proc. Soc. Exptl. Biol. Med.*, **29**, 1169 (1932).

The following table is convenient in calculating standard clearance. It gives values of $\sqrt{V}$.

V *ml. per minute*	$\sqrt{V}$	V *ml. per minute*	$\sqrt{V}$	V *ml. per minute*	$\sqrt{V}$	V *ml. per minute*	$\sqrt{V}$
0.2	0.45	0.7	0.84	1.2	1.10	1.7	1.30
0.3	0.55	0.8	0.89	1.3	1.14	1.8	1.34
0.4	0.63	0.9	0.95	1.4	1.18	1.9	1.38
0.5	0.71	1.0	1.00	1.5	1.23	2.0	1.42
0.6	0.78	1.1	1.05	1.6	1.27	2.1	1.45

Interpretation. In patients with diminishing renal function, the blood-urea clearance shows evidence of diminution sooner than does the blood-creatinine content, the blood-urea content considered without relation to urea excretion, or the phenolsulfonephthalein excretion. The blood-urea clearance usually falls below 50 per cent of normal values before any of the other three show abnormality. Only after the blood-urea clearance indicates less than 20 per cent of normal renal function are all values for blood urea, creatinine, and phenolsulfonephthalein found outside the limits of normal variation. The maximum clearance is normally about 40 per cent greater than the standard clearance, the mean values being 75 ml. (variations 64 to 99 ml.) of blood per minute for the maximum and 54 ml. (variations of 40 to 68 ml.) for the standard. The method is based on the view that with abundant urine the urea excretion per minute equals the urea contained in a constant volume of blood.

2. Phenolsulfonephthalein Test: Principle. This test for renal function was devised by Rowntree and Geraghty. It depends upon the injection into the tissues of a dyestuff which is eliminated rapidly by the normal kidneys, and can be easily estimated quantitatively in the urine.

This dyestuff, phenolsulfonephthalein, is nonirritative to the body either when taken by mouth or when injected into the tissues, so that it does no harm to an already weakened kidney.

The patient upon whom the test is to be performed is given 300 to 400 ml. of water 20 to 30 minutes previously, in order to assure a free flow of urine. Just before the start of the test, the bladder is emptied and the urine discarded.

Procedure. **One ml. of a solution containing 6 mg. of phenolsulfonephthalein[250] per ml. is injected intramuscularly in the lumbar region, the time of injection being noted. The patient is then catheterized and the urine as it forms thereafter allowed to drop into a beaker containing 2 drops of 25 per cent NaOH. The appearance of a red color in the alkalinized urine indicates beginning excretion of the drug, the normal time being within 5 to 10 minutes after its injection. Urine is now collected in one-hour samples. In**

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[250] This solution is prepared by adding 0.6 g. of phenolsulfonephthalein and 0.84 ml. of 2 N NaOH to enough 0.75 per cent NaCl solution to make 100 ml. This gives the monosodium or acid salt which is slightly irritant locally when injected. It is necessary to add two to three drops more 2 N NaOH which changes the color to a Bordeaux red. This preparation is nonirritant. Suitable preparations of the dye in sterile ampuls may be obtained from pharmaceutical supply houses.

patients with obstruction to the flow of urine from the bladder, the retention catheter is stoppered and the urine drawn off at the end of each hour. Other patients may simply be allowed to urinate at the hourly periods.

To each hour sample of urine is added 25 per cent NaOH, drop by drop, until the maximum intensity of color appears. This color will remain constant for an indefinite period of time. Each sample is then placed in a 1000-ml. volumetric flask and diluted to the mark with distilled water.

Compare the color intensity of each sample either colorimetrically or photometrically against a standard. To prepare the standard, place a sufficient amount of the phenolsulphonephthalein solution to contain 3 mg. of the dye (i.e., one-half of the amount administered) in a beaker, dilute with a little water, and add 25 per cent NaOH dropwise to maximum color intensity. Transfer quantitatively to a 1000-ml. volumetric flask, dilute to the mark with water, and mix.

For colorimetric comparison, match the unknowns against the standard in the usual way. It is convenient to set the standard at 10 mm. For photometric measurement, determine the densities of standard and unknown in a photometer at 520 mμ, setting the photometer to zero density with water.

CALCULATION. *For colorimetric measurement:*

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 50 = \text{per cent of administered dye in sample}$$

For photometric measurement:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}} \times 50 = \text{per cent of administered dye in sample}$$

Interpretation. The amount of the drug eliminated normally is 40 to 60 per cent during the first hour and 20 to 25 per cent during the second hour, or a total of 60 to 85 per cent for two hours. The amount of the drug excreted has been found to be independent of the quantity of urine obtained. Ordinarily, two one-hour samples are sufficient; in case of delayed excretion the collection of hourly samples may be continued until practically all of the drug has been recovered in the urine.

If it is desired to test the function of each kidney separately, ureteral catheterization must be resorted to, the experiment otherwise being performed as above described.

The phenolsulfonephthalein test may be used to indicate the amount of derangement in quantitative functional disturbance of the kidneys, as in chronic interstitial and chronic parenchymatous nephritis or uremia.

It is claimed that the rate of excretion of phenolsulfonephthalein is affected by certain extrarenal factors; namely, the albumin and hydrogen-ion concentrations of the blood.

3. Mosenthal Test for Kidney Function:[251] ***Principle.*** The patient under examination is placed for a day on a more or less definite diet.[252] The urine is collected in six two-hour periods during the day and one 12-hour night period. These urine specimens are analyzed for volume, specific gravity, total nitrogen, and chlorides.

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[251] Mosenthal: *Boston Med. Surg. J.*, **170**, 245 (1914); *Ohio State Med. J.*, **18**, 348 (1922).

[252] A diet suitable to ordinary hospital conditions is given by Kahn: *Functional Diagnosis*, p. 260, New York, W. F. Prior Co., 1920. It is not essential, as was formerly believed, to prescribe a diet abundant in diuretic foods or beverages, since ordinary foods contain sufficient diuretic materials for the proper carrying out of the test. In private practice it is only necessary that the patient eat three full meals a day and record the approximate quantities—1 cup of coffee, 2 slices of toast, 2 tablespoonfuls of oatmeal, etc.

Procedure. **On the day of the test have the patient empty the bladder at 8 A.M. and start the diet for the day which, if desired, may contain approximately 13 to 14 g. of nitrogen, 8 to 9 g. of salt, 1700 to 1800 ml. of fluid, and considerable purine material in meat, soup, tea, and coffee.[253] No solid food nor fluid of any kind must be taken between meals and *especial care must be observed that nothing is eaten or drunk after the evening meal.* The meals should start at 8 A.M., 12 noon, and 5 P.M.**

Collect the urine punctually at the end of every 2-hour period until 8 P.M., and place in separate bottles. Collect the night urine from 8 P.M. to 8 A.M. of the following day in another bottle. Measure the volume of each specimen of urine and determine in each case the specific gravity, total nitrogen, and total chlorides.

Interpretation. The test is of particular value apparently as giving earlier indications of diminished kidney efficiency than is true of some other tests used. It is sometimes difficult to interpret the results obtained in terms of renal involvement because of the influence of possible extrarenal factors. In general, however, the normal response is one in which the specific gravity figures vary at least 9 points (less if too little water is taken) from the highest to the lowest and the volume of the *night* urine is 400 ml.[254] or less. If the percentage of nitrogen and sodium chloride in the night urine or in the highest of any of the day specimens is 1 per cent, a normal condition is indicated. Values under 1 per cent, however, may or may not be abnormal.

Time of Day	*Urine*		*Sodium Chloride*		*Nitrogen*	
	Ml.	*Sp. gr.*	*Per Cent*	*G.*	*Per Cent*	*G.*
8–10	153	1.016	1.32	2.02	0.89	1.26
10–12	156	1.019	1.25	1.95	0.74	1.15
12–2	194	1.012	0.64	1.24	0.59	1.14
2–4	260	1.014	0.77	2.00	0.56	1.46
4–6	114	1.020	0.99	1.13	0.95	1.08
6–8	238	1.010	0.43	1.02	0.52	1.235
Total day	1115	..	..	9.36	..	7.32
Night, 8–8	375	1.020	0.63	2.36	1.23	4.61
Total, 24 hours	1490	..	..	11.72	..	11.93
Intake	1760	..	..	8.50	..	13.40
Balance	+270	..	..	−3.22	..	+1.47

When kidney function becomes involved, the first signs are usually demonstrated in the night urine. The quantity becomes increased and the specific gravity and the nitrogen concentration are lowered. One or all of these changes from the normal may occur. In severe cases of chronic nephritis an advanced degree of functional inadequacy of the kidney is indicated by a markedly fixed and low specific gravity, a diminished output of both salt and nitrogen, a tendency to total polyuria, and a night urine showing an increased volume, low specific gravity, and low concentration of nitrogen. Such functional pictures are, however, not confined to nephritis. They are found frequently in many other conditions: pyelitis, cystitis, hypertrophied prostate, marked anemia, pyelonephritis, polycystic kidney, and diabetes insipidus. The table above taken from Mosenthal shows the response of a normal individual.

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[253] See footnote 252.

[254] This represents the usual normal limit. Volumes in excess of 750 ml. are distinctly abnormal, whereas volumes between 400 and 750 ml. are of doubtful significance.

4. Other Methods. Inulin is filtered through the glomeruli and neither excreted nor reabsorbed by the tubules. This is the basis for an inulin-clearance test for renal (glomerular) function.[255]

The *p*-aminohippuric acid (PAH) clearance test is employed as an index of renal blood flow since this compound is filtered through the glomeruli and excreted via the tubules.[256]

Other kidney function tests based on dye excretion rates or on concentration and dilution efficiencies have been proposed.

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[255] Miller, Alving, and Rubin: *J. Clin. Invest.,* **19,** 89 (1940).

[256] Bull, Joekes, and Lowe: *Clin. Sci.,* **9,** 379 (1950).

32

Isotopes

The chemical properties of the several elements depend upon the number and configuration of the unit negative charges, or *electrons*, which surround the nuclei of their atoms. The number of such planetary electrons in the neutral atom of any given element is equal to the number of *protons*, each having a unit positive charge, in the nucleus of that atom. An element is characterized by a fixed and definite *atomic number* of protons in the nuclei of its atoms, balanced by an equal number of planetary electrons.

All atomic nuclei, with the single exception of the nucleus of ordinary hydrogen, contain one or more *neutrons* in addition to protons. The mass number of the neutron (1.00896) is almost identical with that of the proton (1.00813), based upon the arbitrary designation of 16 for the mass number of the commonest variety of oxygen. The number of neutrons in the nuclei of the atoms of a given element is not necessarily fixed, but may vary within narrow limits. Therefore the atoms of most elements contain nuclei with different numbers of neutrons, and consequently of different mass numbers. Each such atomic species, with a specific mass number as well as a specific atomic number, is called an *isotope*.

The complete symbol for any isotope includes the chemical symbol of the element preceded by the atomic number written as a subscript and followed by the mass number written as a superscript. Thus ordinary oxygen would be written ${}_8O^{16}$, ordinary carbon as ${}_6C^{12}$, and ordinary hydrogen as ${}_1H^1$. These elements also have less abundant isotopes such as ${}_8O^{17}$, ${}_8O^{18}$, ${}_6C^{13}$, ${}_1H^2$, and ${}_1H^3$. Since the atomic number is implicit in the chemical symbol, it is more common practice to omit the subscript and simply write O^{16}, C^{12}, H^1, O^{17}, O^{18}, C^{13}, H^2, or H^3 for the isotopes mentioned in the previous sentences. An equally logical but less often used system is to give the two numbers, omitting the chemical symbol.

Most elements exist in nature as mixtures of isotopes. The table on p. 971 lists the isotopes of the elements of major physiological significance. The proportions of the several isotopes in a naturally occurring sample of an element are, as a general rule, constant regardless of the source of the material. For example, the percentage of O^{17} is the same in atmospheric oxygen and in oxygen obtained by decomposition of ocean water or of silicate or carbonate rocks. Most of the naturally occurring isotopes listed in the table are stable, which means that they do not exhibit radioactivity as a result of spontaneous nuclear decay.

NATURALLY OCCURRING ISOTOPES OF ELEMENTS OF BIOCHEMICAL INTEREST

Element	*Number Protons* (Z)	*Number Neutrons* ($A - Z$)	*Mass No.* (A)	*Per Cent Occurrence within Element*
Hydrogen	1	0	1	99.98
Hydrogen (deuterium)	1	1	2	0.02
Hydrogen (tritium)	1	2	3	trace
Carbon	6	6	12	98.9
Carbon	6	7	13	1.1
Nitrogen	7	7	14	99.62
Nitrogen	7	8	15	0.38
Oxygen	8	8	16	99.76
Oxygen	8	9	17	0.04
Oxygen	8	10	18	0.20
Fluorine	9	10	19	100.00
Sodium	11	12	23	100.00
Magnesium	12	12	24	78.6
Magnesium	12	13	25	10.1
Magnesium	12	14	26	11.2
Phosphorus	15	16	31	100.00
Sulfur	16	16	32	95.06
Sulfur	16	17	33	0.74
Sulfur	16	18	34	4.18
Chlorine	17	18	35	75.4
Chlorine	17	20	37	24.6
Potassium	19	20	39	93.3
Potassium	19	21	40	0.01
Potassium	19	22	41	6.7
Calcium	20	20	40	96.96
Calcium	20	22	42	0.64
Calcium	20	23	43	0.15
Calcium	20	24	44	2.06
Iron	26	28	54	5.84
Iron	26	30	56	91.61
Iron	26	31	57	2.17
Iron	26	32	58	0.31
Cobalt	27	32	59	100.00
Copper	29	34	63	69.1
Copper	29	36	65	30.9
Zinc	30	34	64	48.89
Zinc	30	36	66	27.81
Zinc	30	37	67	4.11
Zinc	30	38	68	18.56
Zinc	30	40	70	0.62
Iodine	53	74	127	100.00

For any given element, with its fixed number of protons, there exists only a limited number of possible isotopes, each with its specific number of neutrons. Of these isotopes, still fewer possess stable nuclei. Among certain elements, usually those containing an odd number of protons, only one of the possible isotopes is stable. Sodium, phosphorus, and iodine are examples of such elements with only one stable isotope. Elements whose nuclei contain an even number of protons are likely to have more than one stable isotope. Tin, for example, has 10. As atomic nuclei increase in size and complexity, the proportion of neutrons required to maintain stability also increases. From the table on p. 971 we can see that iron (atomic number 26) requires 30 neutrons to stabilize its most common isotope; the neutron-proton ratio is 1.15. Iodine (atomic number 53) has for its stable isotope a neutron-proton ratio of 1.40, while the most complex of all stable elements is bismuth (atomic number 83), whose 126 neutrons give it a neutron-proton ratio of 1.52.

All nuclei more complex than that of bismuth, and many that are less complex, are unstable and undergo spontaneous changes in neutron-proton composition. Such changes constitute *radioactivity* and may occur in one or more of three ways:

Alpha particles, each consisting of 2 protons and 2 neutrons, may be emitted, resulting in a loss of 4 in mass number and of 2 in atomic number.

Beta particles, which are high-speed electrons, may be emitted, causing a gain of 1 in atomic number by the change of a neutron into a proton, and no change in mass number.

Gamma rays, which are electromagnetic radiations comparable to x-rays but more energetic and penetrating, may be emitted and cause no change in mass or atomic number. The production of γ rays, and the high velocities at which α and β particles travel, are evidence of the release of *nuclear energy* previously stored in the bonds between protons and neutrons. Such nuclear energy is of an order of magnitude sufficient to permit its evaluation in terms of mass, according to the relationship demonstrated by Einstein, that 9×10^{20} ergs of energy are equivalent to 1 gram of mass.

When these energetic radiations impinge upon surrounding atoms or molecules, they not only produce chemical changes by altering the configuration of planetary electrons, but they also cause changes in nuclear structure, transmuting one element into another. The cyclotron and the atomic pile are devices for the production of high-energy radiations in high concentrations. By their use, radioactive isotopes can be prepared in useful quantities. Radioactive isotopes are now known for every chemical element. Both types of isotopes, stable and radioactive, have been useful in biological investigation. The two types require different procedures for their utilization and have different applicabilities. Each type will be considered separately.

RADIOACTIVE ISOTOPES

Radioactive isotopes differ with regard to availability, half-life, and type of radiation emitted. Availability now appears to be largely a matter of technical development. The type of radiation emitted (kind and intensity) influences the sensitivity and methods of measurement. The half-life of a radioactive element is the time required for one-half of the atoms to disintegrate. Isotopes with a very short or very long half-life are of less value than those with a half-life of the order of weeks or months. A short half-life means that too great a proportion of the original material will have lost its activity (and original chemical nature) during the time required for transport after preparation to the place of use, and for the manipulative details of an experiment. A very long half-life means that a high proportion of the isotope must be incorporated into a compound if accurate radioactivity measurements are to be made.

In contrast to the ordinary radioactive half-life defined in the preceding paragraph, we sometimes speak of the *biological half-life*, which is the time taken for a radioactive element within the body of a given organism to lose half of its activity. Elements which are firmly fixed in the body, such as plutonium in bone, have a biological half-life comparable to the ordinary half-life. Excretion of other radioactive elements causes a loss in total body radioactivity frequently more significant than the loss by radioactive decay.

Measurement of Radioactivity. Most methods for the measurement of radioactivity depend upon the fact that energetic α or β particles can convert atoms or molecules of a gas to positive ions by displacing electrons from them. Each positive ion so formed is paired either with the free detached electron or with a negative ion formed by the union of the electron with another neutral atom or molecule. The number of such ion pairs formed per centimeter of the path of a particle is called its *specific ionization*. Gases can also be ionized by γ rays, which detach high velocity electrons from atoms or molecules in their path. These secondary electrons in turn produce ion pairs.

The ionization chamber can be used for the measurement of any type of ionizing radiation, but is particularly adaptable to α particle counting. In its simplest form, an ionization chamber consists of two parallel conducting plates, usually about 1 cm. apart, mounted in a chamber containing a suitable gas, with provision for maintaining a potential difference across the plates and for measuring the current flow through the circuit. The radioactive material can be placed on the lower plate or alternatively the α particles may enter the chamber through a thin window of mica. No current flows unless ionization of the gas is produced, when positive and negative ions will travel towards the oppositely charged plates. Within the range of voltage across the plates suitable for the individual instrument (usually 100 to 500 volts), every ion formed reaches the plates and causes an electrical impulse. The instrument is designed to take advantage of the high specific ionization of α particles and to minimize the disadvantage of their short range. The ionizing chamber is usually used in connection with an amplifier and a mechanical counter, or a counting-rate meter. A *scaler* is a device used with mechanical counters to cut down, by a predetermined factor, the number of electrical impulses recorded. A *pulse analyzer* or "kicksorter" differs from a scaler in that it transmits only stronger impulses, eliminating the weak effects of β and γ radiation, and may also assort the transmitted impulses according to strength and allot them to different counting circuits. In counting mixed radiations, α particles may be screened out by a thin sheet of aluminum, and both α and β particles by a thin sheet of lead.

In *proportional counters* the applied voltage is increased to a range (500 to 800 volts) where the electrons liberated in the primary ionization are given sufficient velocity to cause secondary ionization with formation of more electrons, which in turn are speeded up in the high-potential field, and so on. This results in a self-amplification of impulses known as the *Townsend cascade*, or *Townsend avalanche*. The degree of such self-amplification depends upon the applied voltage.

The Geiger-Müller counter utilizes a still higher voltage (800 to 1500 volts) with a greater degree of self-amplification and only minimal variation with small changes in applied voltage. The cascades produced under these conditions are likely to continue for some time after the entry of the initiating ionizing radiation, and so may merge with the impulses from subsequent rays or particles. To resolve individual impulses, some form of quenching is necessary, which may be internal by the use of polyatomic organic gases, or external by the use of resistances. An adequately quenched Geiger-Müller counter will resolve individual particles at rates less than 5000 per second. The probability of two or more impulses occurring during an interval too short for resolution can be calculated—the *coincidence correction*—for a given tube and a given counting rate. Geiger-Müller counters have been designed for all types of ionizing radiations, but are most suitable for β particles and γ rays.

Scintillation counters utilize the flashes of light produced, chiefly by α particles, upon impact of the radiation upon zinc sulfide and certain other substances. The photomultiplier tube is used in counting circuits based on this principle. Still other types of counters are actuated by the change in electrical conductivity of certain crystals when exposed to ionizing radiations.

The activity of any radioactive source can be expressed in *curies*. The curie was originally defined as the quantity of radon in radioactive equilibrium with one gram of radium. It is now defined by the approximate equivalent, 3.7×10^{10} disintegrations per second. A millicurie, therefore, describes a radioactive source with 3.7×10^{7}, and a microcurie such a source with 3.7×10^{4} disintegrations per second. Another unit, the *rutherford*, defines the quantity of radioactive material that decays at a rate of 10^{6} disintegrations per second. Note that these units refer to disintegrations, and not to particles or rays emitted. From measurements of radiation per unit time, expression of the results in curies or rutherfords requires a calculation of disintegrations per unit time utilizing the decay scheme (which may be quite complex) of the radioactive element in question. By measuring the ionization produced by any γ ray emitter, its source strength can be expressed in *roentgens per hour at one meter* (see p. 976) without knowledge of the disintegration scheme.

Radioautography is a method for detecting and measuring radioactive substances by the effect of their radiations upon the silver bromide of photographic emulsions. The action of light and of ionizing radiations upon the emulsion is comparable in that electrons are transferred from bromide ions to silver ions, with formation of metallic silver—the latent image—around which the silver produced by further reduction in the developing process precipitates. A *radioautograph* is therefore produced by placing the radioactive material in close contact with a photographic emulsion. Light must of course be excluded, and direct chemical action of the specimen upon the photographic emulsion may be obviated by interposing a thin layer of celloidin. Following adequate exposure, the photographic emulsion is developed, fixed, washed, and dried in the manner customary for ordinary photographic negatives. In histological studies, it is advantageous to have stained section and radioautograph superimposed for observation. Several techniques for this purpose have been described.[1] Figure 258 shows by radioautographs the comparative secre-

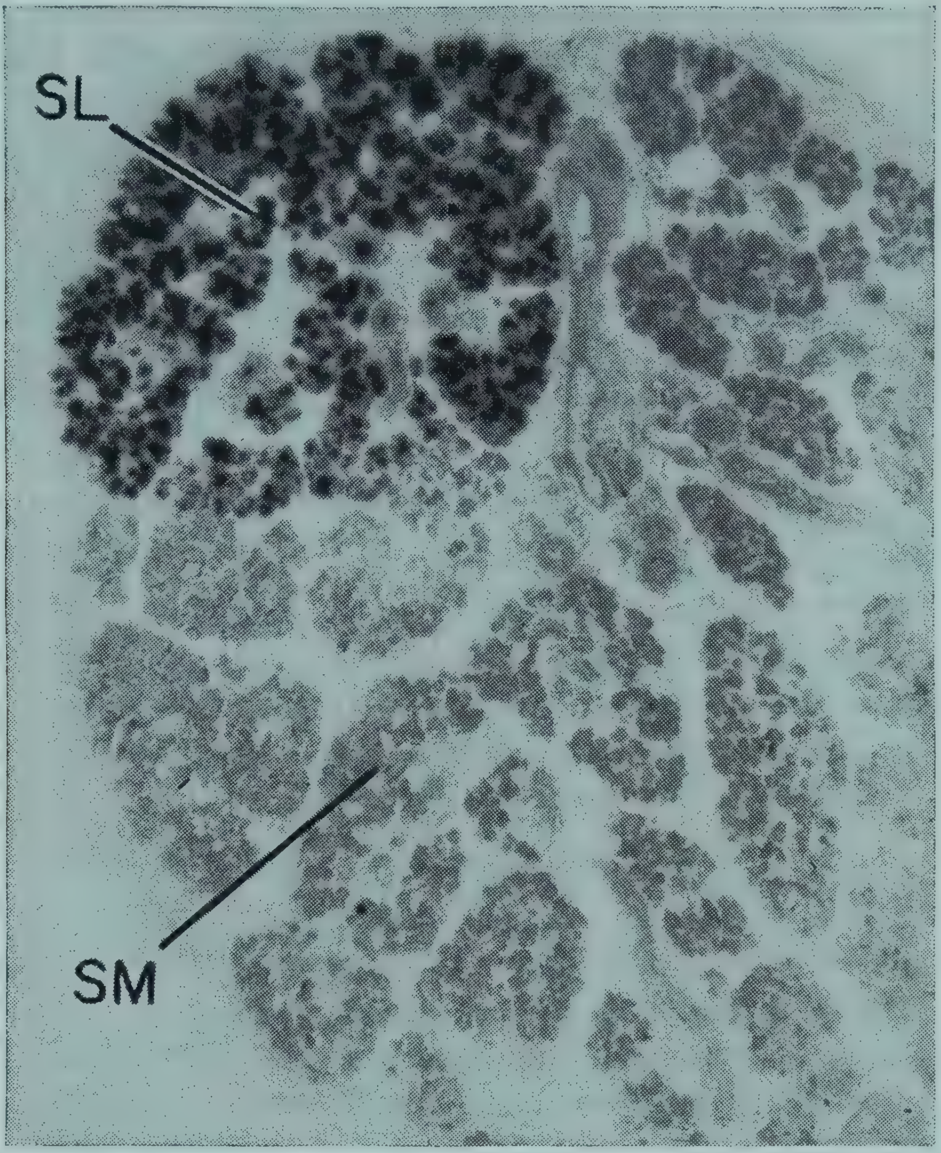

FIG. 258. RADIO-AUTOGRAPHS OF SUBLINGUAL (*SL*) AND SUBMAXILLARY (*SM*) GLANDS OF A RAT GIVEN AN INJECTION OF C^{14}-LABELED BICARBONATE IMMEDIATELY AFTER BIRTH.

The dark area indicates the presence of C^{14}-labeled substances as seen in an animal sacrificed 4 hours after injection.[1a]

Courtesy, R. C. Greulich and C. P. Leblond.

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[1] Gross, Bogoroch, Nadler, and Leblond: *Am. J. Roentgenol. Radium Therapy*, **65**, 420 (1951).

[1a] The sublingual gland (darker area) shows an intense reaction given by the mucous material present in the acinar cells as well as the lumen of the ducts. The elaboration and release of the sublingual mucus is extremely rapid. The submaxillary secretion is considerably slower.

tory activity of the sublingual and the submaxillary glands in newborn rats.

Biological Effects of Ionizing Radiations. The unit in which exposure to x-rays or to γ rays is measured is the *roentgen*. The roentgen (r) is defined as the quantity of x-rays or γ rays which will produce, by ionization, 1 electrostatic unit of electricity of either sign in 1 ml. of dry air measured under standard conditions of temperature and pressure. A more convenient equivalent definition for the roentgen is the amount of x-rays or γ rays which must be absorbed by 1 g. of air to cause it to gain 83.8 ergs of energy.

To include other types of ionizing radiations, such as α and β particles, the *roentgen equivalent physical* or *rep* is defined as the quantity of ionizing radiation of any kind which, if absorbed by the soft tissues of the body, causes an energy gain per gram of tissue equivalent to that caused by 1 r of x-rays or γ rays.

Since the absorption of 1 rep of one type of radiation might have a biological effect quite different quantitatively from that of 1 rep of another type, the *roentgen equivalent man* or *rem* is defined as the quantity of radiation of any type which produces the same amount of damage in man upon absorption as 1 r of x-rays or γ rays. For x-rays, γ rays, and β particles, 1 rep equals approximately 1 rem, but for α particles, 1 rep equals 10 to 20 rem.

The combined experience and experiments of medical radiologists and of the physicists charged with the protection of workers handling radioactive materials has led to the tentative conclusion that the *total permissible dose* of all kinds of ionizing radiation is 0.3 rem per week over the whole body. In defining this dose, it is understood that very concentrated whole body exposure for short times is dangerous even within the limits of the permitted amount per week. Larger doses, up to 1.5 rem per week, may be permitted if exposure is limited to hands and forearms.

Neutrons are emitted at high velocity from atomic explosions and in nuclear reactors. Such "fast neutrons" possess high energies but limited powers of penetration. When fast neutrons interact with tissue, they are first slowed down by collisions with nuclei, predominantly those of hydrogen, which are thereby detached from their molecules and stripped of orbital electrons. In this manner, fast neutrons are actually ionizing radiations. When the velocity of the neutrons is cut down by repeated collisions, the neutrons may be captured by ordinary atomic nuclei, bringing about nuclear transformations. Neutron capture may result in the ejection from the nucleus of energetic protons, or in the emission of γ rays accompanying the formation of deuterium in the case of hydrogen, and in other instances the formation of radioactive isotopes. All of these nuclear transformations are further sources of ionizing radiation, which are spoken of collectively as *induced radioactivity*.

Since water is the substance present in greatest amount in the majority of living cells and organisms, the effect of ionizing radiations upon water is of considerable importance. Detachment of an electron from water leaves a positive water ion (H_2O^+) which can react with an intact water molecule to yield a hydrated hydrogen ion and a free hydroxyl radical.[2]

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[2] Weiss: *Nature*, **153**, 748 (1944).

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH$$

The detached electron can also react with a water molecule, yielding a hydroxyl ion and a hydrogen atom:

$$e^- + H_2O \rightarrow OH^- + H$$

The ions react together to form water molecules again. Some of the hydrogen atoms unite to form hydrogen molecules. Some of the free hydroxyl radicals unite to form hydrogen peroxide. A hydrogen atom may unite with a hydroxyl radical to form water. Thus radiation of pure water forms hydrogen and hydrogen peroxide.

If the water contains dissolved oxygen, it will combine with hydrogen atoms, increasing the yield of hydrogen peroxide, and forming perhydroxyl radicals:

$$H + O_2 \rightarrow HO_2$$

Among the substances which have been shown to diminish the destructive effect of ionizing radiations upon solutions of enzymes or upon living cells, cysteine and other substances containing the sulfhydryl group are noteworthy. The sulfhydryl group can reduce hydroxyl radicals, hydrogen peroxide, and perhydroxyl radicals,[3] thus removing from aqueous solutions these products of ionizing radiation. The other product, hydrogen atoms, is removed indirectly through the formation of perhydroxyl radicals (in the presence of dissolved oxygen, see equation above).

The lethal effects of radiation result chiefly from the destruction of the more susceptible cells (all types of blood and epithelial cells) and the failure to form new cells to replace them. Death after a single heavy exposure is usually delayed weeks or months, during which time blood cell counts fall and ulcerations of epithelia develop. The symptoms which appear promptly after heavy irradiation—the *initial radiation response* or *radiation sickness*—do not necessarily indicate a lethal exposure. Gastrointestinal upset and lowered blood pressure may occur even after heavy x-ray dosage within the therapeutic range, particularly if applied to the abdomen. Possible mechanisms to explain this prompt reaction are the liberation of VDM (to be discussed later) and direct effects of radiation on the autonomic nervous system. The damaging effects of ionizing radiations upon tissues can be demonstrated histologically.[4] The clearest results are obtained in specimens prepared by freezing-drying, then cut and mounted dry. Vacuoles appear within the cell nuclei of tissues which have been subjected to radiation. In cells such as lymphocytes, which are sensitive to radiation, the vacuoles distend and eventually rupture the nucleus. In more resistant cells, the vacuoles formed are smaller, and are later extruded through the nuclear membrane without necessarily destroying the cell.

The well-recognized *genetic effect* of sublethal exposures to ionizing radiations is an increase in the rate of occurrence of mutations. It has not been definitely established whether this effect is chemical (upon the nucleoprotein molecule) or mechanical (upon the chromosome). The genetic effect of radiations has been shown to be cumulative, depending more upon the total radiation received by the experimental plant or animal than upon the intensity of the radiation. Data relating amount of radiation to genetic effects in man are not available. Sterility can be produced by ionizing radiations in sublethal doses.

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[3] Swallow: *J. Chem. Soc.*, 1334 (1952).

[4] Warren, Holt, and Sommers: *Am. J. Clin. Path.*, **22**, 411 (1952).

The amount of energy expended in producing the lethal effects of ionizing radiation in living cells is very small, less than one-millionth of the cell's normal daily expenditure of energy in metabolic processes.[5] It is therefore evident that damage to key substances, such as enzymes or nucleic acids, by ionization must be postulated to explain the destructive action of relatively minute amounts of energy. Sulfhydryl enzymes are particularly susceptible to inactivation by radiation, even *in vitro*.[6] One chemical mechanism by which tissue damage from radiation is known to take place is the formation of peroxides. Two-thirds of the total biological effect of exposure of *Paramecium aurelia* to x-rays could be attributed to peroxide formation in the medium, since it could be abolished by catalase and could be duplicated by adding H_2O_2 to the medium without radiation.[7]

Ionizing radiation also brings about the release within the circulating blood of a toxic substance which causes severe peripheral vasodepression. The behavior of this substance is like that of a similar substance liberated in traumatic shock. This substance was first designated simply as VDM (vasodepressor material), but was later identified as ferritin. By several criteria based upon physiological action, ferritin and the VDM of irradiated animals are identical.[8] A vasoexcitor material which has not been identified chemically, has been reported in situations of reversible shock and in animals that survived into the second week following irradiation.

Radioactive Tracers. Physiological chemists have repeatedly sought a means of placing an identifying tag or label upon a particular sample of a foodstuff, drug, or intermediate and following it through its metabolic transformations in a living organism. Knoop used such a tracer in his studies of fatty-acid oxidation when he substituted the phenyl group on the omega carbon atom of fatty acids. Elaidic acid, the *trans* isomer of oleic acid, has been similarly used. In both these instances, the pathway of the tracer substance was found to be not identical in all metabolic sequences with that of the natural substance under investigation.

The use of tracer substances identical with the corresponding natural substances except for one or more radioactive isotopic atoms in the molecule has obviated this difficulty. The tagged atoms can be identified by their radioactivity, whatever the compounds may be into which they are introduced by metabolic activities. Such tracer substances can be prepared by ordinary chemical synthesis, or in some instances more efficiently by biosynthesis, using as starting material radioactive isotopes obtained by the use of the cyclotron or the nuclear reactor. The fundamental postulate in metabolic studies with radioactive tracers is that the chemical changes undergone by the radioactively tagged molecule during its metabolism are the same as those of the corresponding untagged natural substance. This postulate is valid if, and only if, two important experimental conditions are maintained: (*a*) radioactivity must be kept at a level low enough not to cause disturbance of cell metabolism, as for example by inactivation of enzymes; and (*b*) the tracer substance must be given in amounts not large enough to exceed physiological concentrations in the organism. The latter condition can be purposely waived if the study involves the effects of unusual concentrations or unphysiological substances.

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[5] Hempelmann, Lisco, and Hoffman: *Ann. Internal Med.*, **36**, 279 (1952).

[6] Barron, Dickman, Muntz and Singer: *J. Gen. Physiol.*, **32**, 537 (1949).

[7] Kimball and Gaither: *Proc. Soc. Exp. Biol. Med.*, **80**, 525 (1952).

[8] Haley: *Trans. N. Y. Acad. Sci.*, Ser. II, **14**, 303 (1952).

Hevesy[9] made the first biological application of radioactive tracers. He followed the absorption and distribution of lead in plants with thorium B (in modern terms, Pb^{212}), a naturally occurring radioactive isotope of lead. The choice of available radioactive isotopes at that time was, of course, extremely limited. Currently the nuclear reactor and the cyclotron make available a large number of radioactive isotopes of elements significant in biology. The radioactive isotopes most useful in present metabolic studies are C^{14}, H^3, P^{32}, Na^{24}, I^{131}, Fe^{55}, Fe^{59}, S^{35}, and Co^{60}. Procurement of radioactive isotopes for experimental work requires the authorization of the Atomic Energy Commission. No useful radioactive isotopes exist for two very important elements, oxygen and nitrogen. Tracer work with these elements must therefore be done with the stable isotopes, O^{18} and N^{15}.

Although samples of pure radioactive isotopes can be isolated (with considerable difficulty), the material used for preparations of tracer substances is almost invariably a mixture of the desired radioactive isotope with stable isotopes of the same element. The *specific activity* of a sample is defined as the number of atoms of the specified radioactive isotope divided by the total number of atoms of the same element. (Unfortunately, the term *specific activity* is also used to mean curies per gram of the element specified. The reader must distinguish these two different quantities by the units used.)

An important consideration enters here into the interpretation of results obtained with labeled organic molecules. If the isotopic element is in a labile position—i.e., if it can enter readily into exchange reactions with the solvent or with other elements in the molecule—interpretation is obscured. For example, an organic acid labeled with deuterium in the ionizable portion of the carboxyl group may exchange with the nonisotopic hydrogen of water present to render the labeling valueless as a means of identifying the compound:

$$R{\cdot}COOD + H_2O \rightleftarrows R{\cdot}COOH + D_2O$$

Thus a stably bound isotope is of more value in this connection than a labile one, and due consideration must be given to this fact. It should be pointed out that some positions in organic molecules are stable *in vitro* but may become labile *in vivo*. Thus the α-amino nitrogen of an amino acid may be stable, but may become detached by metabolic processes so that the remainder of the molecule is no longer identifiable in terms of isotope content:

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[9] Hevesy: *Biochem. J.*, **17**, 439 (1923).

$$R\cdot CHN^{15}H_2\cdot COOH \rightarrow R\cdot CO\cdot COOH + N^{15}H_3$$

Thus, although this type of labeling permits following the course of the nitrogen metabolism, it is valueless as a guide to what happens to the remainder of the molecule after deamination; for this purpose, the carbon chain should contain isotopic carbon, preferably in as many positions as possible, or stably bound deuterium may be present.

Measurements of blood volume and extracellular fluid volume have long been made by nonisotopic dilution methods involving the injection of some substance foreign but harmless to the body. A known amount was injected, time allowed for equilibration, and the concentration measured in the blood plasma. With a substance such as the dyestuff T 1824 (Evans blue), which escapes but slowly from the blood vascular system, a close estimate of blood plasma volume is possible. With soluble bromides or thiocyanates, which diffuse through all extracellular fluids but do not significantly enter cells, extracellular fluid volume can be measured. For this latter type of measurement the use of radioactive Br^- permits the use of very small doses and avoids the pharmacological effects of this ion. For blood volume determinations, the use of red cells tagged with radioactive phosphorus obviates the errors inherent in the dye method. For measurement of total body water, deuterium oxide is the indicator of first choice, since it mixes freely with body water and traverses all cell boundaries. Its chief disadvantage is the special instrumentation required for its measurement (see "Stable Isotopes," below). Heavy water is toxic, but not in the low concentrations (0.2 atoms per cent deuterium) used in such studies. A minor error arises from exchange of deuterium and from its incorporation into organic molecules. This error has been estimated at less than 1.5 per cent.[10]

EXPERIMENTS ON RADIOACTIVE ISOTOPES

Determination of Blood Volume:[11] ***Principle.*** The determination of blood volume in rabbits is accomplished by incubating blood cells from the animal with 80 to 100 microcuries of P^{32} labeled phosphate for 45 to 60 minutes at 37° C. Approximately 10 per cent of this activity is absorbed by the cells, which are then washed, reconstituted to volume, and injected into the animal. After an interval for distribution of the labeled cells throughout the circulation, a blood sample is withdrawn for counting. The dilution of the activity of the cells (the volume of the whole blood) is obtained by dividing the activity of the injected cells by that of the blood withdrawn.

The relative simplicity of the experiment allows for total working space on one 24-inch by 30-inch table. This is covered with a large blotter so that any spilled active material may be readily discarded. Special apparatus consists of a basic scaler and Geiger-Müller counter with a shielded manual sample changer. The counting apparatus is kept on a table separate from the working area. The radiation hazard is minimal to the animal or to the experimenters, as shown by Reid and Orr,[12] who give data for 100 humans with average uptake of the cells amounting to 7 per cent of the 50 to 100 microcuries incubated, with the largest single uptake 19.5 per cent. In calculating the hazard when the activity was injected into a human, they assumed a 20 per cent uptake in a 50-kg. individual. This would calculate to 0.07 rep for approximately one week, which would diminish to 0.035 rep in two weeks (14.3 days half-life). This calculated value is deliberately high, for it assumes no excretion, but is yet a small fraction of the normally permissible 0.3 rem. With but small precaution, therefore, no radiation hazard is encountered. Indeed, it is probable that the students are exposed to greater dangers in their other experiments by handling corrosive acids and hot solutions. As a means of illustrating fully the manner of working with isotopes, students may characterize the radiophosphorus they use by calibrating its millicurie strength against a set of simulated reference standards. They may also count a sample daily for two minutes, for seven or more successive days, in order to calculate the half-life of the P^{32} used and thus identify it. This also affords a check on the purity of the isotope shipment, and acquaints the student with the main remaining features of low-energy isotope experimentation.

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[10] Edelman, Olney, James, Brooks, and Moore: *Science*, **115**, 447 (1952).

[11] Kelly, Simonsen, and Elman: *J. Clin. Invest.*, **27**, 795 (1948), modified for use with rabbits by Dr. Fabian Lionetti.

Procedure. **All glassware should be siliconed and dried. To all Cutler or similar tubes, a pinch of heparin (gauge by tip of spatula) should be added.**

(a) PREPARATION OF TAGGED CELLS. **To two 15-ml. centrifuge tubes approximately 1.5 to 2 ml. of acid-citrate-dextrose solution (ACD)[13] is added. Using a syringe wet with ACD, 20 to 30 ml. of blood is taken from a rabbit by cardiac puncture. Between 5 and 8 ml. of the blood is placed in one and the rest in the other centrifuge tube. These are covered with parafilm and inverted 20 to 30 times to prevent clotting. Both tubes are placed in the incubator at 37° C. until ready for use, and inverted intermittently. To the tube containing 5 to 8 ml. blood, add 80 to 100 microcuries P^{32} in volume less than 1 ml. (to maintain isotonicity). The tubes are inverted 5 to 10 times and returned to the incubator for 45 to 60 minutes. Invert the tubes 10 times approximately every 5 minutes. Care is required to avoid shaking the tubes. At the end of the incubation time, the cells are spun down in a refrigerated centrifuge at 2000 r.p.m. for 15 minutes. The fluid is removed with a capillary pipet and replaced with cold ACD to the original volume. Recentrifuging and reconstituting to volume is repeated 3 times, mixing being done by careful inversion 20 to 25 times. If slight hemolysis is observed during the washing, it is advisable to eliminate the final one or two washings. After removal of the third washing, the volume is replaced to original with cold ACD. The tube is inverted gently 20 to 25 times or more, and placed in the icebox until ready for use.**

(b) PREPARATION OF THE TAGGED CELLS FOR COUNTING INITIAL ACTIVITY. **Since the tagged cells are too active to count conveniently, they are diluted serially with inert blood. Into each of 4 small test tubes containing heparin place 3 ml. of blood. Care is required to prevent clotting. The pipet should be previously rinsed with ACD. To the first tube is carefully added exactly 1 ml. of the tagged red cells (making sure that the cells are well mixed before the addition). The tagged cells in the first tube have thus been diluted 4 times.**

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[12] Reid and Orr: *J. Clin. Invest.*, **29**, 313 (1950).

[13] *ACD (Acid-Citrate-Dextrose Solution).*

Trisodium citrate	2.20 per cent (w/v)
Citric acid	0.80 per cent
Anhydrous dextrose	2.25 per cent

It is advisable to make up the solution in 500-ml. lots, and to keep it stoppered and refrigerated.

One ml. of the 1:4 suspension in the first tube is now removed and added to the second tube containing 3 ml. of untagged blood; this gives a 1:16 dilution. Transfers are continued until the fourth tube is prepared containing a 1:256 dilution of the initial 1 ml. of tagged cells. Mix carefully by drawing blood into and expelling it from the pipet 15 to 20 times. A pair of 1-ml. portions of the final dilution is placed in the planchet for counting. To each is added a drop of 1 per cent detergent solution (Duponol) to facilitate spreading in the planchet.

(c) INJECTION OF RADIOACTIVE CELLS. The rabbit is weighed and placed in a box with a cover so notched as to allow the head and ears to protrude. Both ears are shaved along the marginal ear veins. The tagged cells are removed from the icebox and inverted to mix them well. One ml. is drawn into a tuberculin syringe and all air bubbles are removed. Holding the right ear tightly, introduce the needle into the right marginal vein. Once in the vein, the needle is extended to the hilt. If the needle slides easily with little friction, the left thumb is placed over the needle and held securely, in order not to withdraw the needle if the animal moves its head. Inject the cells slowly. They are infused readily if the needle is in the vein. The needle is then withdrawn and the site clamped with the bulldog clamp. After 20 minutes of equilibration in the rabbit, a sample is obtained for counting by making a nick in the left marginal ear vein. Preliminary treatment with xylol or toluene on the surface of the ear may be used to promote a more rapid flow. Several ml. of blood are collected in a heparinized tube and 1 ml. is pipetted into a planchet. Five drops of Duponol (ca. 1 per cent) is added. The planchet of diluted tagged cells (1:256) and the one prepared from the animal are incubated at 37° C. to dryness (overnight). A third sample of untagged whole blood is similarly prepared for taking background counts. If it is necessary to save time, the wet samples may be counted, but the counts are less reproducible and less accurate.

(d) PACKED CELL VOLUME. This part of the procedure is necessary only if plasma volume is to be determined. The plasma volume is found from the whole-blood volume by a hematocrit determination, finding the plasma fraction of the whole-blood volume. The tube with the remainder of the blood is spun at 2500 to 3000 r.p.m. for 60 minutes. The top level of the plasma at the meniscus is read. The level of the packed red cells is noted, ignoring the buffy coat. The second decimal is estimated in the reading.

CALCULATION. The instrument background B is measured in counts per minute, using the sample of inert blood. The background count B is subtracted from the counts of both the experimental and injected samples. The experimental samples withdrawn from the animal are counted for a time interval corresponding to several thousand counts, and the activity in counts per minute is recorded (E), after subtracting B. The activity of the injected sample I is found from the count (corrected for background) of the diluted tagged blood D multiplied by the dilution factor 256, thus

$$I = D \times 256$$

The whole-blood volume (WBV) in milliliters is therefore

$$\mathrm{WBV} = \frac{I}{E}$$

RADIOACTIVE ISOTOPES IN MEDICINE

Ionizing radiation has been applied with increasing skill and success to destroy tumors or other undesired tissues. Radioactive isotopes in general are applicable as sources of ionizing radiation for this purpose. Certain radioactive isotopes are concentrated by physiological mechanisms in certain cells or tissues—I^{131} in the thyroid, P^{32} in blood cells—just as the normal stable isotopes of these elements are so concentrated. In this manner, ionizing radiation can be localized to certain tissues for their destruction when they have become neoplastic. After administration of P^{32} by mouth or intravenously (in the form of a phosphate), it is taken up first by the red cells, with concentrations in white cells increasing after the second day. In leukemic patients who have received P^{32}, after one week the isotope concentration in leukocytes is four to five times that in red cells.[14]

Smaller doses of radioactive isotopes may be used in diagnostic studies. The amount of I^{131} in the thyroid gland of a human patient can be estimated by a measurement made externally over the thyroid region by a 4-tube Geiger-Müller counter.[15] If an oral tracer dose containing 100 to 150 microcuries of I^{131} is given, 30 or 40 per cent of it should be demonstrable in the thyroid gland on the next day in patients with normal thyroids. Hyperthyroidism is characterized by higher uptake of iodine—around 70 per cent, while in hypothyroidism the uptake is low—around 12 per cent as measured under similar conditions. Administration of I^{131} in larger amounts is utilized for the destruction by radiation of cancerous thyroid tissue, or in reducing the activity of the gland in hyperthyroid patients. This form of treatment brings about results comparable to surgical removal of the thyroid, in whole or in part according to the dosage of the radioactive material.

STABLE ISOTOPES

Most of the common elements as ordinarily encountered in nature consist of mixtures of stable isotopes. Thus ordinary oxygen consists of the three stable isotopes O^{16}, O^{17}, and O^{18}, in the proportion respectively of 99.76, 0.04, and 0.20 per cent. More than 200 stable isotopes of the various elements have been recognized as existing, but only a very few have been obtained in concentrated form. To obtain the separate isotopes from a mixture, or fractions relatively enriched with respect to one isotope, advantage is taken of properties such as diffusion or reaction velocity which may vary with mass differences. Thus a water molecule containing O^{18} will be heavier than one containing O^{16}, and the two types may be separated by controlled fractional distillation. By such methods or their equivalent, the stable isotopes H^2 (deuterium), C^{13}, N^{15}, O^{18}, and S^{34} have been made available for biological investigation. The first of these to become available in amount sufficient for investigative purposes was deuterium, first obtained by Washburn and Urey in 1932 by the fractional electrolysis of water. It was in fact the availability of deuterium which suggested to the imaginative genius of Schoenheimer and Rittenberg the possibility of its use in biological investigations, a concept which may be said to have initiated the present phase of application of isotopes to biological problems.

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[14] Duffy and Howland: *N. Y. State J. Med.*, **52**, 551 (1952).

[15] Freedberg, Chamovitz, and Kurland: *Metabolism*, **1**, 26 (1952).

The chief application of the stable isotopes in biological investigation is for the purpose of labeling or tagging a molecule or part of a molecule. The stable isotopes have the advantage over the radioactive type in that the time of preparation of the compound incorporating the isotope, or the duration of the experiment, are of no importance; there is no question concerning the possible effect of radiation on the experiment; and in some instances the isotope may be recovered at the end of the experiment and used again. Disadvantages relative to radioactive isotopes include the increased difficulty of measurement; the necessity for a higher concentration, since measurements of radioactivity are far more sensitive than measurements of mass; and the relatively few stable isotopes available.

With the exception of deuterium, the stable isotopes are measured with an instrument called the mass spectrometer. The principle upon which this instrument operates is illustrated diagramatically in Fig. 259. The sample in the form of a gas, e.g. N_2, CO_2, is admitted to a highly evacuated chamber across which a stream of electrons is flowing. An electron colliding with a gas molecule causes it to become ionized, e.g. $CO_2 \rightarrow CO_2^+$. The gas ions so formed are accelerated by suitable means to produce a stream of ions which passes along the tube shown and through a powerful magnetic field, so arranged as to deflect the ion from its initial path. On leaving the magnetic field, the ions pass to a collector plate and produce an ion current which is what is actually measured as an index of the number of ions striking the plate. Ions of different mass, e.g. $C^{12}O_2^+$ and $C^{13}O_2^+$, may be differentiated by either varying the strength of the magnetic field, or more commonly this strength is held constant and the accelerating voltage is varied. Results are obtained in the form of a graph relating ion current to accelerating voltage. Isotopic ions differ on such a graph in their position along the voltage scale (which can be converted into mass, since a constant relation exists between these two quantities); the ratio of one isotope to another is given by the ratio of ion currents at the respective points on the mass (voltage) scale. It is this ratio of isotopes which is ordinarily desired—i.e., the relative abundance for example of C^{12} over C^{13} in the sample as compared to their relative amounts in ordinary nonisotopic compounds.

The measurement of deuterium is a special case. It may of course be measured with the mass spectrometer, but equally satisfactory results can be obtained by converting the deuterium to the form of water (as by burning the sample in oxygen) and determining the specific gravity of the water so produced. There is a predictable relationship between the specific gravity of the water and its content of *heavy water*, D_2O. A common method for determining specific gravity is the *falling drop method*, in which the time required for a drop of water to fall through a definite distance in a nonaqueous solvent is measured. This method is capable of high accuracy.

The isotope content of compounds labeled with the stable isotopes usually is expressed in terms of *atoms per cent* of isotope. For example, if a substance contains 12 hydrogen atoms, and one of these is replaced by deuterium, the isotopic content will be 1/12, or 8.3 atoms per cent.

This is subject to some correction, since an ordinary "nonisotopic" compound contains a certain proportion of isotopes in accordance with their normal abundance. The excess of a given isotope over that normally present is referred to as *atoms per cent excess*, a more satisfactory term when dealing with variations in the isotope content of compounds.

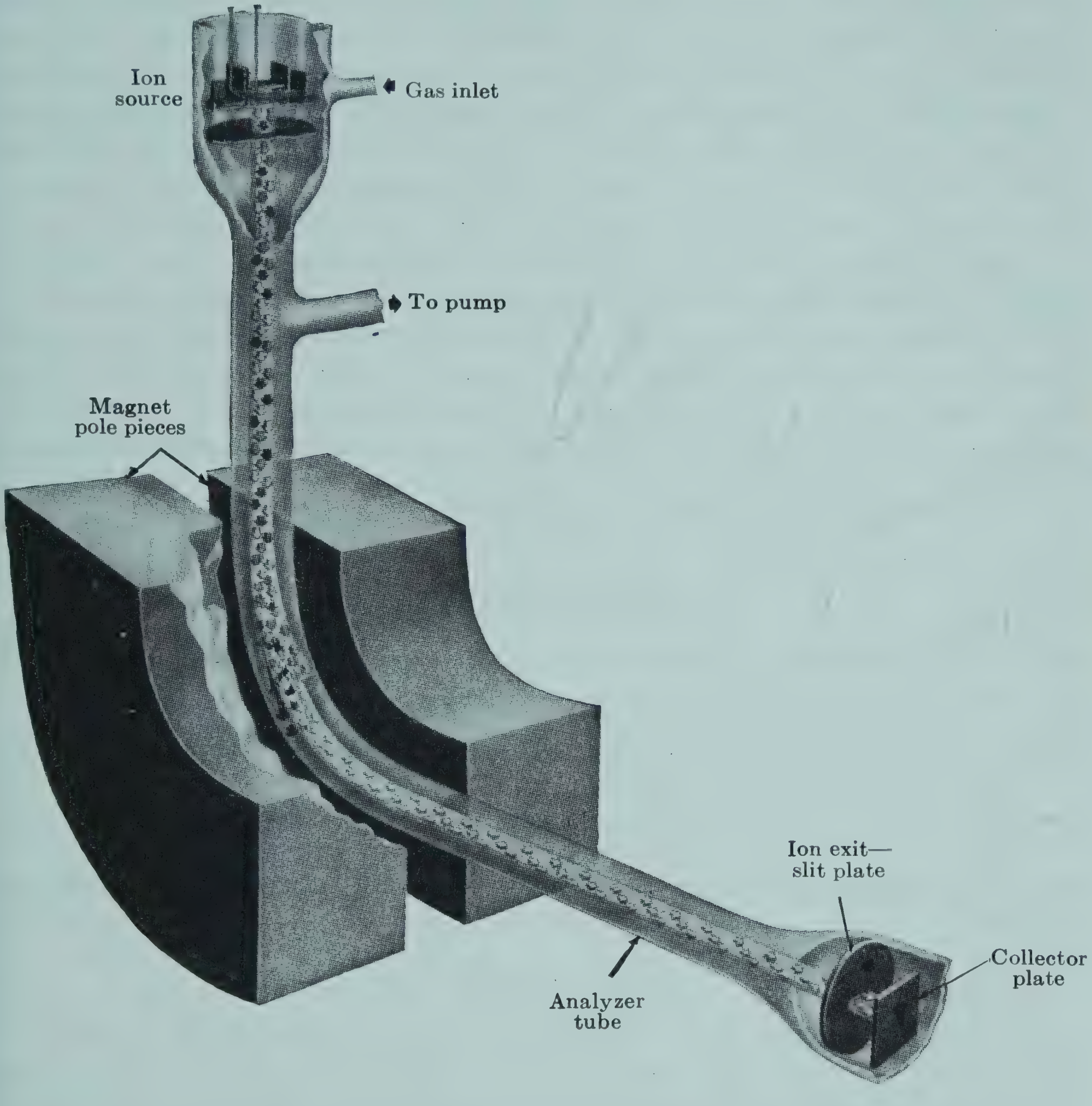

FIG. 259. OPERATION OF THE MASS SPECTROMETER TUBE: IDEALIZED VIEW.

The ion beam is projected into the tube and deflected by the magnetic field. The separation of the ions of different masses is indicated by the variance in the radius of curvature of the gray- and black-arrow beams in the area of the magnetic field.

Courtesy, Westinghouse *Engineer*.

A special problem arises in connection with the use of deuterium in metabolic tracer studies. Deuterium has twice the atomic weight of ordinary hydrogen, and hence diffuses more slowly and will slow down reaction rates. Differences in atomic weight exist (by definition) among all isotopes of a given element, but in no other instance is the difference nearly as great proportionately.

Analysis by Isotope Dilution. Stable or radioactive isotopes may be used in chemical analysis in instances where other methods fail or account of lack of specificity, as for example in the assay of culture filtrates of *Penicillium chrysogenum* for penicillin G to the exclusion of other penicillins. Four steps are involved in such an isotope dilution assay: (*a*) a tracer sample of the compound to be determined (e.g., penicillin G) is prepared with a known isotopic content, and with the isotopic atom in a position which will not exchange; (*b*) a known quantity of this isotopically tagged compound is isolated in a state of purity, but not necessarily quantitatively; (*c*) the isotopic content of the isolated sample is determined. By comparison of this figure with the isotopic content of the tracer sample prepared in step (*a*), and knowing the proportions in which the mixture in step (*b*) was made, the quantity of the substance in the unknown sample (e.g., penicillin G in the culture filtrate) can be immediately calculated. If the isolated sample has the same isotopic content as the tracer sample, the quantity in the unknown was zero. If the isolated sample has half the isotopic content as the tracer, the quantity in the unknown was equal to that in the tracer added; if one-third, equal to twice that added; one-fourth, equal to three times that added, and so on. In general, if the isotopic content of the isolated sample is x per cent of the original, the quantity in the unknown is $\frac{100 - x}{x}$ of the quantity added.

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33

Carbohydrate, Fat, and Protein Metabolism

GENERAL

The components of a normal mixed diet which supply all the requirements of the animal body for growth and maintenance include proteins, fats, carbohydrates, the various vitamins, water, and certain inorganic elements. In the present chapter are considered some of the nutritional requirements for proteins, fats, and carbohydrates, and the changes which these substances undergo subsequent to their digestion and absorption into the animal body. The energy metabolism of these substances has been considered in Chapter 25. Brief discussions concerning certain metabolic phases are also found in the chapters dealing with the urinary constituents and in other connections. The questions of mineral metabolism, water, and the vitamins are discussed in subsequent chapters.

Throughout the discussions which follow, frequent mention will be made of the use of isotopes, which have proved such valuable tools in the study of intermediary metabolism and nutrition. The isotopes themselves and their use in biology are discussed in Chapter 32. Adequate entrance to the voluminous literature of intermediary metabolism and nutrition is afforded by the footnote references and the reviews and special articles listed in the bibliography at the end of this chapter.

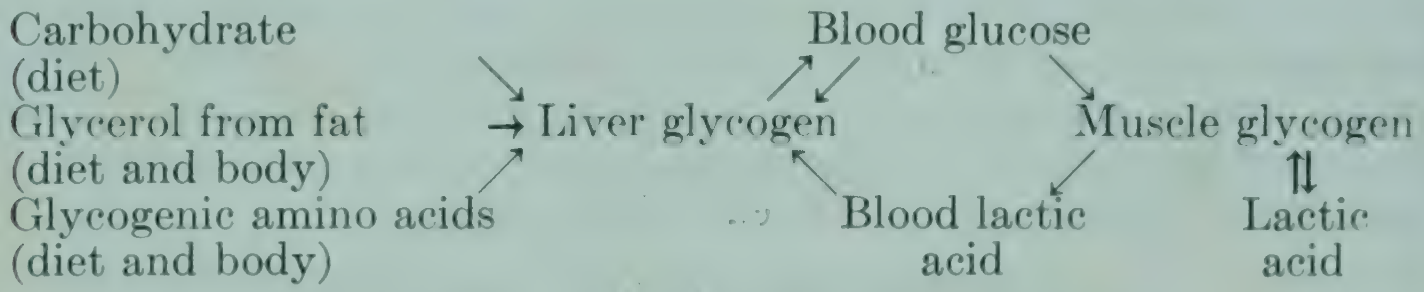
CARBOHYDRATE METABOLISM

Carbohydrates supply the major portion of the daily energy requirements of the normal individual; on an ordinary diet more than half of the total daily calories usually come from this source. In addition to being oxidized as a source of energy, carbohydrates may be transformed to glycogen, supply the carbon chain for certain amino acids, or be converted into fat. Of these various processes, glycogen formation and breakdown appears to occupy a central position.

Glycogen Formation and Breakdown (Glycogenesis and Glycogenolysis). Glycogen is found in practically all of the tissues of the body, but in varying amounts; the glycogen content of the brain, for example, is so low as to require special care for its detection. The muscles and liver contain most of the glycogen of the body, largely because of their bulk relative to that of the other tissues. Glycogens from various tissues—e.g., muscle glycogen, liver glycogen—appear to be chemically similar if not identical but to have markedly different physiological significance. Thus the glycogen content of muscle is relatively constant in the absence of exhaustive muscular contraction and is little affected by starvation or the nature of the diet, which may produce variation in the liver glycogen content from a mere trace to upward of 8 per cent of the weight of this organ.

Glycogen, therefore, may have both a functional and a storage significance. The capacity of the animal to store carbohydrate as glycogen is relatively limited, however, as contrasted with its capacity to store fat. The adult human body may contain about 300 g. of glycogen, of which only that fraction found in the liver (normally about one-half of the total) represents a significant source of carbohydrate for general metabolic purposes. Fasting for a few days is usually sufficient to deplete the animal of all available stored carbohydrate. Any carbohydrate metabolism in a fasting animal after the exhaustion of stored carbohydrate is due presumably to synthesis of carbohydrate from noncarbohydrate precursors (gluconeogenesis).

Major precursors of liver glycogen within the animal body include the glucose, fructose, and galactose (but not pentose) produced by carbohydrate digestion in the intestinal tract; the glucose and lactate of the blood; the glycerol portion of the fats of either the diet or the body; and certain amino acid constituents of the proteins of either the diet or the tissues which are capable of being converted into glucose and hence into glycogen. Precursors of muscle glycogen include the glucose of the blood and, to an extent which is still debatable, the lactic acid produced from glycogen itself during muscle contraction. These various interrelationships with regard to liver and muscle glycogen may be summarized in the following diagram, adapted from Cori:



Knowledge that a particular substance is a precursor of liver glycogen, for example, usually has been obtained by demonstrating that an increased liver glycogen content follows the feeding of the test substance. Perfusion of the isolated organ and incubation with surviving liver slices also have been used. It has generally been assumed that if glycogen formation occurred, the test substance was directly incorporated into the newly formed glycogen. That the situation is not quite as simple as this has been unequivocally demonstrated by the use of glycogen precursors labeled with radioactive carbon. For example, if lactate labeled with radioactive carbon is fed to animals whose liver glycogen has been depleted by fasting, and the newly formed glycogen which results from the lactate feeding is subsequently isolated, only a fraction of this glycogen proves to contain the isotope label. Furthermore, the isotope is found distributed among the various carbon atoms of the glucose residues in the labeled glycogen in such a way as to indicate that the fed lactate had entered into other reactions before some of the carbon atoms became incorporated into glycogen. Similar results have been obtained with glucogenic amino acids labeled with isotopic carbon. If glycogen formation is stimulated by glucose or lactate feeding in the presence of radioactive carbon dioxide (as bicarbonate), the radioactive carbon is also found in the glycogen subsequently isolated. Thus glycogen formation and breakdown is a complicated and continuous process within the body; according to Stetten,[1] the half-life of rat-liver glycogen is but one day. The accumulation of glycogen in the presence of an apparent precursor may be due only in part to direct utilization of the precursor itself, or may even represent a "sparing" action on glycogen metabolism without any direct connection with such metabolism.

Knowledge concerning the intermediate steps in glycogen formation and breakdown has been obtained largely with tissue preparations or isolated enzyme systems. Much of this work has been done with either muscle or liver; it is believed that conclusions drawn from such studies are generally applicable, in principle if not in specific detail. The various reactions known to be concerned in glycogen formation and breakdown may be divided into two phases, anaerobic and aerobic, summarized as follows:

$$\begin{tikzcd}
 & \text{Glycogen} + \text{Phosphate} \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \\
 & \text{Glucose-1-phosphate} \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \\
\text{Glucose} + \text{ATP} \arrow[r] & \text{Glucose-6-phosphate} \arrow[r] \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \text{Glucose} + \text{Phosphate} \\
\text{Fructose} + \text{ATP} \arrow[r] & \text{Fructose-6-phosphate} \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \\
 & \text{Fructose-1,6-diphosphate} \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \\
 & \text{Triose phosphates} \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \\
 & \text{Phosphoglycerate} \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \\
 & \text{Phosphopyruvate} \arrow[d, leftarrow, shift left] \arrow[d, shift right] & \\
\text{Lactate} \arrow[r, shift left] \arrow[r, leftarrow, shift right] & \text{Pyruvate} \arrow[d] & \text{Anaerobic phase} \\
 & CO_2 + H_2O & \text{Aerobic phase}
\end{tikzcd}$$

Many of these reactions already have been discussed in detail in Chapter 10 in connection with the chemistry of lactic acid formation in muscle, and the chemical structures of the various intermediate compounds also will be found there.

It will be noted that the immediate chemical precursor of glycogen is the compound glucose-1-phosphate (the *Cori ester*). The enzyme catalyzing the reversible reaction between glycogen, inorganic phosphate, and glucose-1-phosphate is known as *phosphorylase*. The equilibrium in this reaction is in favor of glycogen; the direction of reaction appears to be determined largely by the concentration of inorganic phosphate. In the presence of excess inorganic phosphate, glycogen breakdown occurs; for glycogen synthesis, it is necessary to keep the inorganic phosphate content low. This apparently is done in the cell by oxidative processes which incorporate the phosphate into phosphate esters, some of which are intermediates in the series of reactions shown. There is thus in effect a *phosphate cycle* (Cori), whereby inorganic phosphate liberated by glycogen formation is used to phosphorylate other compounds which, in turn, can give rise to glycogen and inorganic phosphate again.

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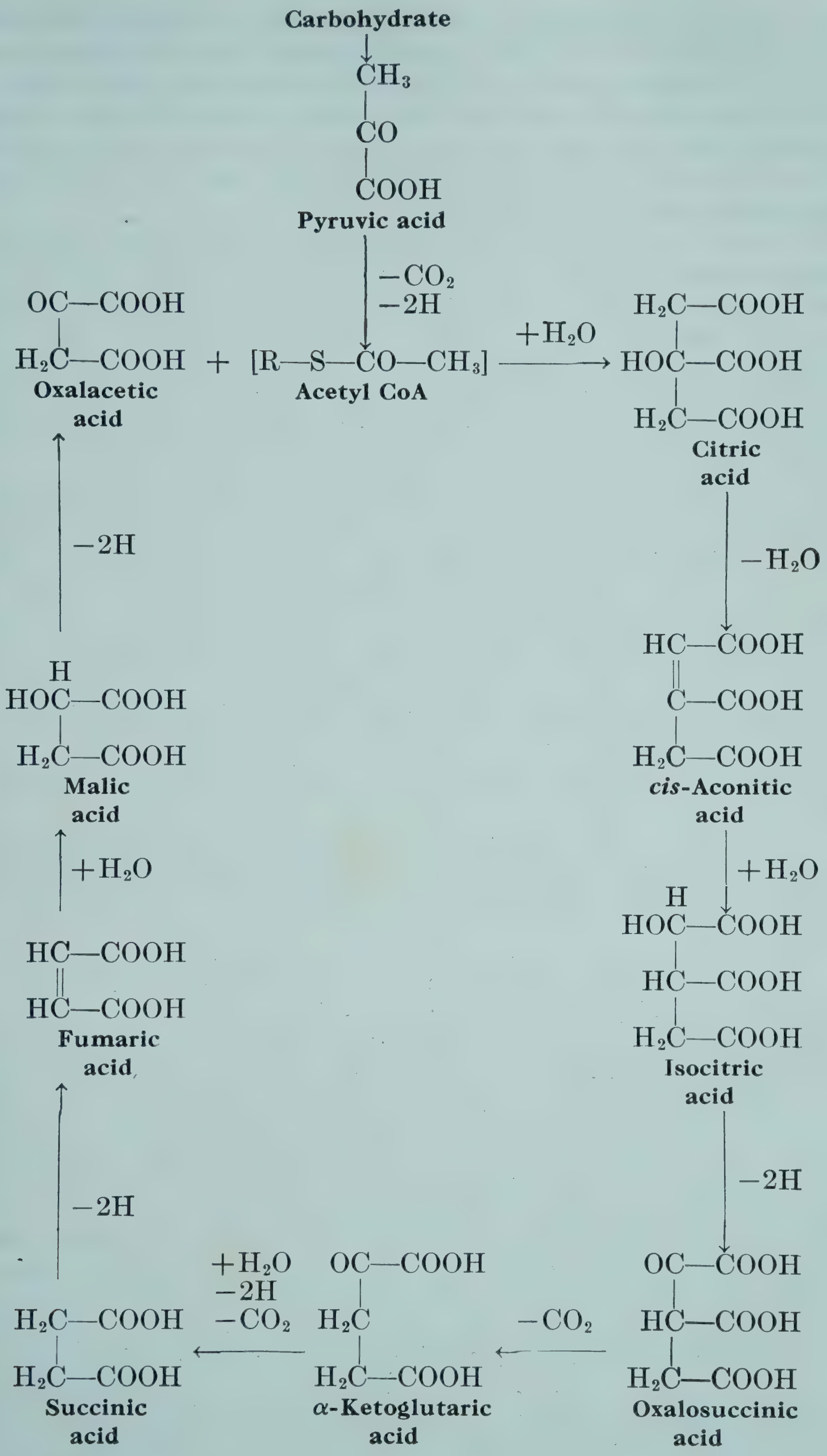
[1] Stetten and Boxer: *J. Biol. Chem.*, 155, 231, 237 (1944).

For free glucose (or fructose) to be converted into glycogen by the reactions shown, phosphorylation is necessary. This phosphorylation—leading to the formation of either glucose-6-phosphate or fructose-6-phosphate, as the case may be—requires the presence of the "high energy phosphate" of adenosinetriphosphate (ATP), and of enzymes of the hexokinase type. In liver these enzymes are known as glucokinase and fructokinase; similar enzymes are found in muscle. In brain tissue there appears to be only one enzyme which, like yeast hexokinase, acts upon both glucose and fructose. The hexokinase enzymes, or at any rate their activities, are apparently under the control of certain hormones, as discussed on p. 1000, and knowledge concerning this control may prove to be of fundamental importance in understanding certain phases of carbohydrate metabolism.

After phosphorylation, glucose becomes available for either glycogen formation, reconversion to glucose, or breakdown to lactic acid anaerobically or complete oxidation aerobically. Reconversion to glucose is due to the enzyme phosphatase, which catalyzes hydrolysis of glucose-6-phosphate to glucose and inorganic phosphate. This is believed to be the origin of the glucose of the blood from the glycogen of the liver (and possibly also of the kidneys). In muscle tissue, which contains no phosphatase, glucose-6-phosphate is either converted to glycogen or degraded to form lactic acid anaerobically or completely oxidized aerobically. The formation of lactic acid from glycogen requires no oxygen; the oxidation of triosephosphate to phosphoglycerate is coupled with the simultaneous reduction of pyruvate to lactate, through the mediation of coenzyme I which is alternately reduced and oxidized, as described in Chapters 10 and 35.

Aerobic Oxidation of Carbohydrate. The precise mechanism whereby carbohydrate is completely oxidized to CO_2 and H_2O is still a subject of discussion. It is felt by many that a reasonable answer to the mechanism of aerobic carbohydrate oxidation is afforded by the cyclic process first described in its essential principles by Krebs and called by him the *citric acid cycle*, later modified by Krebs and others and now more generally known as the *tricarboxylic acid cycle*.

According to this concept, carbohydrate is considered to be degraded to the stage of pyruvic acid, $CH_3COCOOH$, by the series of reactions just described for the anerobic breakdown; but whereas under anaerobic conditions pyruvic acid is converted to lactic acid as the chief end product of the process, under aerobic conditions the pyruvic acid undergoes a different series of reactions. These reactions of the tricarboxylic acid cycle are summarized in the accompanying diagram, which for purposes of clarity has been considerably simplified, as will be pointed out.


Carbohydrate
CH_3
CO
COOH
Pyruvic acid
$-CO_2$
$-2H$
OC—COOH
H_2C—COOH
Oxalacetic acid
+
[R—S—CO—CH_3]
Acetyl CoA
$+H_2O$
H_2C—COOH
HOC—COOH
H_2C—COOH
Citric acid
$-H_2O$
HC—COOH
C—COOH
H_2C—COOH
cis-Aconitic acid
$+H_2O$
H
HOC—COOH
HC—COOH
H_2C—COOH
Isocitric acid
$-2H$
OC—COOH
HC—COOH
H_2C—COOH
Oxalosuccinic acid
$-CO_2$
OC—COOH
H_2C
H_2C—COOH
α-Ketoglutaric acid
$+H_2O$
$-2H$
$-CO_2$
H_2C—COOH
H_2C—COOH
Succinic acid
$-2H$
HC—COOH
HC—COOH
Fumaric acid
$+H_2O$
H
HOC—COOH
H_2C—COOH
Malic acid
$-2H$

As shown in this diagram, the first major reaction in the aerobic oxidation of pyruvic acid is the production of citric acid from pyruvic acid and oxalacetic acid. Earlier doubts concerning the role of citric acid itself in the citric acid cycle have now been resolved, and there is general agreement as to its importance as indicated. Oxalacetic acid is normally present in small amount in actively respiring tissue, and is constantly being regenerated, as will be shown.

The exact mechanism of the formation of citric acid from pyruvic and oxalacetic acids has been the subject of intensive study. Earlier views, based on a variety of evidence, postulated the presence of a reactive 2-carbon fragment (active acetate), formed from pyruvic acid, which condensed with oxalacetic acid to form citric acid. Active acetate has now been identified by Lynen as the S-acetyl ester of coenzyme A, acetyl CoA, symbolized as follows: $R—S—CO—CH_3$, where R is the remainder of the coenzyme A molecule (see pp. 1190–1.

The mechanism of formation of acetyl CoA from pyruvic acid in animal tissues is not entirely clear in all its phases, but there is no doubt that it occurs; in fact, it is believed that all substances entering the citric acid cycle at the 2-carbon fragment level must be precursors of acetyl CoA. One method of formation of acetyl CoA from pyruvic acid which has been established for heart tissue by Korkes *et al.*[2] is the reaction between pyruvic acid, coenzyme A, and diphosphopyridine nucleotide (DPN, coenzyme I), in the presence of diphosphothiamine (cocarboxylase), to give acetyl CoA, CO_2, and reduced DPN:

$$\text{Pyruvic acid} + \text{DPN} + \text{CoA} \rightarrow \text{Acetyl CoA} + CO_2 + \text{DPNH} + H^+$$

In the presence of the condensing enzyme, which is the first enzyme of the citric acid cycle to be isolated from animal tissues in crystalline form (Ochoa), acetyl CoA readily condenses with oxalacetic acid to form citric acid and CoA:

$$\text{Acetyl CoA} + \text{Oxalacetic acid} \rightarrow \text{Citric acid} + \text{CoA}$$

Thus the over-all reaction results in the formation of citric acid from pyruvic acid (by way of acetyl CoA) and oxalacetic acid. The role of DPN is that of hydrogen acceptor; in the living cell the hydrogen is ultimately transferred to oxygen. The role of cocarboxylase is not entirely clear. A similar oxidation of pyruvic acid can be demonstrated in certain bacterial extracts; here the cocarboxylase is said to be associated with 6,8-thioctic acid (see Chapter 35) in its action.

Citric acid is readily metabolized in muscle and other animal tissues through the stages of *cis*-aconitic acid and isocitric acid as shown, in the presence of the enzyme aconitase, which mediates an equilibrium among the three compounds. Isocitric acid once formed undergoes oxidation by dehydrogenation in the presence of isocitric dehydrogenase to yield oxalosuccinic acid, which is then decarboxylated to give α-ketoglutaric acid. It is not clear whether an oxalosuccinic decarboxylase is concerned here, or the entire reaction from isocitric acid to α-ketoglutaric acid is mediated by isocitric dehydrogenase alone. It is known however that triphosphopyridine nucleotide (TPN, coenzyme II) is coupled in these reactions, so that the oxidation of isocitric acid involves the reduction of TPN. The over-all reaction at this particular step is therefore as follows:

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[2] Korkes, del Campillo, and Ochoa: *J. Biol. Chem.*, 195, 541 (1952).

$$\text{Isocitric acid} + \text{TPN} \rightleftarrows \alpha\text{-Ketoglutaric acid} + CO_2 + \text{TPNH} + H^+$$

As in the previous reaction involving DPN, the hydrogen of the reduced TPN is ultimately transferred to oxygen to form water.

The substance α-ketoglutaric acid is readily converted by decarboxylation and dehydrogenation (with DPN as hydrogen acceptor) to give the C_4 dicarboxylic acid succinic acid. The intermediate stages in this reaction are not entirely clear, but they appear to involve the formation of succinyl CoA as an intermediate. Succinic acid is then acted upon by the enzyme succinic dehydrogenase, present in most animal tissues, to give fumaric acid by oxidative dehydrogenation. Fumaric acid readily undergoes hydration in the presence of the enzyme fumarase to form malic acid, which on oxidation in the presence of the enzyme malic dehydrogenase yields oxalacetic acid. At this point the entrance of another molecule of pyruvic acid permits the entire cycle to be repeated.

Thus the net result of the cycle is the complete disappearance of one molecule of pyruvic acid, giving rise to three molecules of CO_2 in the process and requiring the ultimate presence of five oxygen atoms ($2.5O_2$) to accept the 10 hydrogen atoms removed by the various dehydrogenation reactions. As indicated above, the mechanism of hydrogen transport to oxygen is by way of the di- and triphosphopyridine nucleotides (coenzymes I and II), the flavoproteins, the cytochromes, and cytochrome oxidase, as described in Chapter 12, "Enzymes."

The over-all reaction is therefore:

$$\underset{\textbf{Pyruvic acid}}{CH_3\cdot CO\cdot COOH} + 2.5O_2 = 3CO_2 + 2H_2O$$

with a respiratory quotient (R.Q.) of 3/2.5 = 1.2. By the combination of reactions described, involving the formation of pyruvate from carbohydrate and the participation of pyruvate in the tricarboxylic acid cycle, it is possible therefore to account for the complete oxidation of carbohydrate to CO_2 and H_2O. To obtain an R.Q. of 1.0, which is the R.Q. of carbohydrate oxidation, the transfer of two more hydrogen atoms to an oxygen atom must enter into the picture. These two hydrogen atoms are presumably those which arise in the oxidation of triosephosphate during the anaerobic phase of carbohydrate breakdown (see p. 274), and which produce lactic acid from pyruvic acid under these conditions. In aerobic metabolism they are transferred to oxygen by way of diphosphopyridine nucleotide (DPN).

The major evidence in favor of such a cyclic process as that described may be summarized as follows: the various postulated intermediates usually increase the respiratory rate of suitable tissue preparations (e.g., minced muscle) when added in catalytic amounts; many of the individual enzymes concerned have been isolated and shown to function as indicated; certain of the postulated intermediates have been isolated from tissue systems in the presence of specific poisons such as malonate and arsenite; and critical examination of the theory by the use of isotopes as markers for specific portions of the various molecules concerned as yet has not revealed any major discrepancies.

The reactions of the tricarboxylic acid cycle afford an explanation for the participation in the processes of carbohydrate oxidation of the various intermediates shown, even though such intermediates are not carbohydrates or may arise from noncarbohydrate precursors. The implications of this relationship to the metabolism of fats and amino acids will be evident from the subsequent discussion in this chapter.

Most of the reactions of the tricarboxylic acid cycle are reversible. An explanation is afforded for the synthesis of carbohydrates from noncarbohydrate precursors which are, or give rise to, components of the cycle, provided a means is available for their conversion to pyruvate. There is ample evidence that such formation of pyruvate occurs in animal tissues. Pyruvic acid may arise by the direct decarboxylation of oxalacetic acid:

$$\underset{\textbf{Oxalacetic acid}}{COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH} \rightarrow \underset{\textbf{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + CO_2$$

This reaction is known to occur in animal tissues. Once formed, pyruvic acid is readily phosphorylated in the presence of ATP:

$$\text{Pyruvate} + \text{ATP} \rightleftarrows \text{Phosphopyruvate} + \text{ADP}$$

Phosphopyruvate then can serve as a direct precursor of the hexoses and glycogen by the reversal of the glycolytic reactions already described on p. 989. Kalckar has shown that phosphopyruvate itself is formed during the aerobic oxidation of the C_4 dicarboxylic acids by tissue extracts; this may arise by the decarboxylation of phosphooxalacetic acid (Lipmann):

$$\underset{\substack{\textbf{Phosphooxalacetic acid}\\ \textit{(enol form)}}}{COOH{\cdot}CH{:}CO(PO_3H_2){\cdot}COOH} \rightarrow \underset{\substack{\textbf{Phosphopyruvic acid}\\ \textit{(enol form)}}}{CH_2{:}CO(PO_3H_2){\cdot}COOH} + CO_2$$

Other reactions doubtless remain to be discovered, but at any rate it is clear that the pathways of carbohydrate synthesis from noncarbohydrate precursors are open by the reactions described.

The possible reversibility of the decarboxylation reactions in the citric acid cycle has stimulated interest in the mechanisms of CO_2 fixation, i.e., the utilization of the carbon of carbon dioxide for metabolic purposes. That CO_2 fixation occurs elsewhere than in green plants and photosynthetic bacteria was first shown for nonphotosynthetic bacteria by Wood and Werkman, who postulated that the reaction involved the formation of oxalacetic acid from pyruvic acid and carbon dioxide:

$$\underset{\textbf{Pyruvic acid}}{CH_3{\cdot}CO{\cdot}COOH} + CO_2 \rightarrow \underset{\textbf{Oxalacetic acid}}{COOH{\cdot}CH_2{\cdot}CO{\cdot}COOH}$$

Proof that CO_2 fixation occurs in animal tissues was afforded by Evans and Slotin[3] using bicarbonate containing radioactive carbon; the α-ketoglutaric acid synthesized by pigeon-liver preparations in the presence of pyruvate and the isotopic bicarbonate was found to contain a significant amount of the isotopic carbon. The carbon of radioactive sodium bicarbonate administered to rats is also found in the glycogen subsequently isolated from the liver.[4] When fatty acids are oxidized to acetoacetate by liver preparations in the presence of radioactive carbon dioxide, the isotope is found incorporated, to a small but significant extent, in the carboxyl group of the acetoacetate formed.[5] Other examples could be cited; they all demonstrate that the release of carbon dioxide by animal tissues is not an exclusively one-way process.

Although the Wood-Werkman reaction has been much studied in connection with CO_2 fixation by animal tissues, there is no good evidence that it occurs elsewhere than in bacteria. Of greater interest in this connection is the reaction discovered by Ochoa in liver extracts, in which malic acid is reversibly oxidized and decarboxylated to give pyruvic acid and CO_2. This reaction requires TPN as hydrogen acceptor, and is catalyzed by the *malic enzyme:*

$$\text{Malic acid} + \text{TPN} \rightleftarrows \text{Pyruvic acid} + CO_2 + \text{TPNH} + H^+$$

If this system is coupled with another TPN-requiring system, such as the combination of glucose-6-phosphate and glucose-6-phosphate dehydrogenase, the TPN may be removed from the system to such an extent that the reaction proceeds to the left and there is a net synthesis of malic acid from pyruvic acid and carbon dioxide.

It is of interest to note here that Ochoa had previously shown that the oxidative decarboxylation of isocitric acid to form α-ketoglutaric acid was experimentally reversible under the proper conditions; however, the equilibrium conditions for the malic enzyme are stated to be 15 times more favorable for CO_2 fixation by the malic enzyme reaction than for the isocitrate-ketoglutarate reaction. It is to be noted that, once malic acid is formed from pyruvic acid and carbon dioxide, the presence of malic dehydrogenase in a tissue will lead to the production of oxalacetic acid. Thus the malic enzyme provides a mechanism for the entry of pyruvic acid into the citric acid cycle other than by way of acetyl CoA.

Despite the undoubted demonstration of its existence, the significance of CO_2 fixation by animal tissues remains obscure. The extent of fixation is usually quite small, since equilibrium conditions ordinarily favor decarboxylation. The comparative ability of various animal tissues to fix CO_2 has been studied by Crane and Ball.[6] According to Ochoa, the malic enzyme reaction provides the only means thus far known whereby a net synthesis of dicarboxylic acids can be obtained from pyruvic acid and carbon dioxide. It has been suggested that CO_2 fixation provides for the constant presence of the catalytic amounts of dicarboxylic acids necessary for the continuous operation of the citric acid cycle. It is interesting to recall here that, as discussed in Chapter 2, the assimilation of CO_2 by green plants during photosynthesis leads to the formation of phosphoglyceric and phosphopyruvic acids, and that malic acid is a subsequent product of the reaction. Thus the fundamental processes of CO_2 assimilation in plant and animal tissues may well be similar.

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[3] Evans and Slotin: *J. Biol. Chem.*, **136**, 301 (1940).

[4] Solomon, Vennesland, Klemperer, Buchanan, and Hastings: *J. Biol. Chem.*, **140**, 171 (1941).

[5] Plaut and Lardy: *J. Biol. Chem.*, **192**, 435 (1951).

[6] Crane and Ball: *J. Biol. Chem.*, **188**, 819 (1951).

Despite the extensive knowledge which has been obtained concerning intermediary carbohydrate metabolism, there is much that is still obscure. The aerobic oxidation of lactic acid, for example, usually is considered to occur by way of conversion to pyruvic acid which is then oxidized by the processes just described. There is some evidence that other as yet unknown and possibly important pathways exist. In certain tissues, glucose can be shown to be oxidizable to form gluconic or phosphogluconic acid, and the latter has been shown to be converted into ribose phosphate, one of the building blocks of nucleic acids. The conversion of carbohydrate to pyruvate and the reactions of the tricarboxylic acid cycle have been studied largely in one tissue, i.e., muscle, and the conclusions derived are not necessarily applicable *in toto* to other tissues. Yet Krebs has pointed out that the various animal tissues thus far investigated possess enzymes similar in general to those required for the tricarboxylic acid cycle, so that while individual differences between tissues doubtless occur, the basic reactions could be fundamentally similar in all tissues.

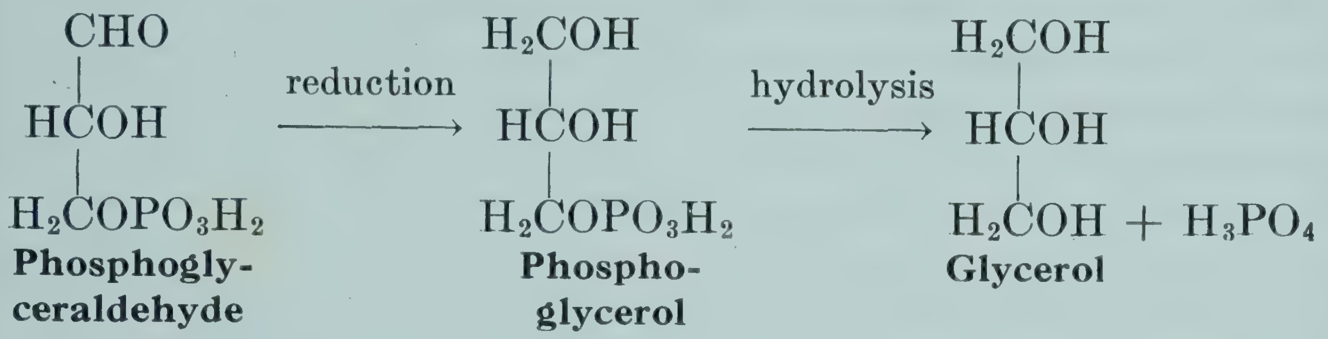
Formation of Carbohydrate from Amino Acids. The reactions of the tricarboxylic acid cycle afford a reasonable explanation for the ability of certain amino acids to give rise to glucose or extra glycogen in the animal body. Thus the amino acids glutamic acid, aspartic acid, and alanine, by metabolic deamination, give rise to α-ketoglutaric acid, oxalacetic acid, and pyruvic acid, respectively. These three latter compounds are recognized components of the tricarboxylic acid cycle.

glutamic acid → α-ketoglutaric acid
aspartic acid → oxalacetic acid
alanine → pyruvic acid

After deamination, therefore, these three amino acid constituents of the protein molecule become indistinguishable from carbohydrate metabolites. Since the reactions of the tricarboxylic acid cycle are reversible, a mechanism is available for either the synthesis of carbohydrate or metabolism via carbohydrate pathways for these amino acids, and for any others which are convertible into these amino acids by metabolic processes. That pathways exist other than those described is quite probable, but they remain to be discovered.

Conversion of Carbohydrate to Fat. The fattening of farm animals and the development of obesity in man on high carbohydrate diets suggest that carbohydrate can be converted to fat in the animal body. In feeding experiments in which a control animal is killed and analyzed for fat while a litter mate is fed a high carbohydrate diet, it can be shown that the latter may, after a time, contain more fat than could be derived from all of the fat and protein fed. In the same way, milch cows without loss of fat from their bodies may secrete in their milk much more fat than can be accounted for by fat and protein ingested. Studies of the respiratory quotient yield similar evidence with regard to the conversion of carbohydrate into fat (see Chapter 24). The availability of isotope-labeled glucose has provided a more direct demonstration of the conversion of carbohydrate to fat. For example, if mice and rats kept on an essentially fat-free diet are given a dose of labeled glucose, between 10 and 15 per cent of the labeled carbon atoms are found in the fatty acids of the animal body after 24 hours.[7] These and other results lead to the conclusion that conversion of carbohydrate to fat does not represent merely the storage of energy, but is also an important pathway in the metabolism of carbohydrate itself.

The glycerol for fat synthesis may arise very easily from carbohydrate, possibly directly from phosphoglyceraldehyde, a normal intermediate in carbohydrate metabolism in tissues:



$$\begin{array}{c}\mathrm{CHO}\\|\\\mathrm{HCOH}\\|\\\mathrm{H_2COPO_3H_2}\end{array}\xrightarrow{\text{reduction}}\begin{array}{c}\mathrm{H_2COH}\\|\\\mathrm{HCOH}\\|\\\mathrm{H_2COPO_3H_2}\end{array}\xrightarrow{\text{hydrolysis}}\begin{array}{c}\mathrm{H_2COH}\\|\\\mathrm{HCOH}\\|\\\mathrm{H_2COH}\end{array}+\mathrm{H_3PO_4}$$

Phosphoglyceraldehyde **Phosphoglycerol** **Glycerol**

The mode of synthesis of the fatty acid portion of the fat molecule is not yet clear. It is generally agreed that the first step in the synthesis involves the formation of reactive 2-carbon fragments, which are then condensed with each other. On the basis of available evidence, the reactive 2-carbon fragments arise by the breakdown of carbohydrate to the stage of pyruvic acid, as described above, followed by oxidative decarboxylation of the pyruvic acid. There is a strong possibility that acetyl CoA is the reactive intermediate in the condensation, although the nature of this condensation has not as yet been precisely defined. Much of the work on fatty acid synthesis from carbohydrate has been done with bacterial systems, and it is not certain to what extent the conclusions reached are applicable to animal tissues. Although fatty acid synthesis has been demonstrated in animal tissue preparations, these preparations are usually too complex to permit a precise explanation of the reaction mechanism.

The formation of neutral fat from carbohydrate by way of pyruvic acid would involve the production of reactive 2-carbon fragments, condensation, reduction of the β-keto compounds formed to saturated acids, and lastly esterification of the saturated acids. Acetoacetic acid itself is known not to be an intermediate in this synthesis, although the possibility remains that an active form of acetoacetic acid, e.g. acetoacetyl CoA, may be concerned. Acetic acid has been shown to be a precursor of the higher fatty acids, presumably after conversion to either acetylphosphate or acetyl CoA; fatty acid synthesis from labeled acetate has been demonstrated in liver homogenates.[8] Moreover, the mammary gland has been shown to be especially active in lipogenesis. Large amounts of acetate arise in ruminants, owing to the fermentation of carbohydrate by microorganisms in the rumen. This acetate is absorbed from the rumen by the blood and carried to the mammary gland, where it is used not only as a source of energy but also for fat synthesis; in fact, Popják[9] reported that 50 per cent of the retained dose of acetate injected into a goat could be accounted for in the milk fat. The relationship of acetic acid to carbohydrate and fat metabolism is further discussed on p. 1009.

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[7] Masoro, Chaikoff, and Dauben: *J. Biol. Chem.*, **179**, 1117 (1949).

The Blood Sugar. The sugar of the blood is glucose, present normally at a concentration of about 80 mg. per 100 ml. of blood, or 0.08 per cent. This level is maintained constant within relatively narrow limits under all ordinary circumstances; significant variations from the normal range usually indicate some aberration of carbohydrate metabolism. The constancy of the blood sugar level apparently is due to a balance between the rate at which glucose enters the blood and the rate at which it leaves the blood. Blood glucose appears to originate chiefly in the liver; the rapid fall in blood glucose level after hepatectomy indicates that other tissues cannot contribute materially to the blood sugar content, although there is some evidence that the kidneys may play a minor role in this respect. The glycogen of the liver usually is considered to be the major source of the blood glucose, but the maintenance of a normal blood sugar level in the fasting animal long after liver glycogen stores are exhausted indicates that carbohydrate synthesis in the liver may also play an important part in this connection.

Factors which lead to a removal of glucose from the blood include oxidation by the tissues, conversion to glycogen in liver and muscle, and to a minor extent in other tissues, storage as fat, and under certain conditions excretion in the urine. Increased oxidation in the tissues and increased conversion to liver and muscle glycogen each tend to lower the blood sugar level; when these two factors operate together, as after the administration of insulin, the resulting hypoglycemia may be profound. In the absence of insulin, as in diabetes mellitus, the inability to store glycogen in the liver coupled with decreased oxidative utilization in the peripheral tissues generally is believed to be responsible for the elevated blood sugar values seen in this condition. An alternate explanation for the hyperglycemia of diabetes mellitus is accelerated gluconeogenesis ("overproduction") rather than impaired oxidative utilization ("underconsumption"). Evidence in favor of the overproduction theory is limited.

Increased glycogenolysis in liver (but not in muscle), as after the administration of adrenaline or during ether anesthesia, likewise causes an elevation of the blood sugar. The glycogen of muscle is not a direct factor in the maintenance of the blood sugar level, but operates only indirectly insofar as the muscles remove glucose for glycogen formation or contribute lactic acid to the blood which is then carried to the liver where blood lactate may be a significant source of liver glycogen and hence of blood glucose.

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[8] Brady and Gurin: *Arch. Biochem. Biophys.*, 34, 221 (1951).

[9] Popják, French, and Folley: *Biochem. J.*, 48, 411 (1951).

Storage of carbohydrate as fat (discussed on p. 996) is apparently a property of most of the tissues of the body. Loss of glucose from the blood because of excretion in the urine will occur whenever the rate of reabsorption of glucose by the renal tubular cells falls behind the rate of entrance of glucose into the glomerular ultrafiltrate—i.e., when the level of plasma glucose exceeds the renal threshold concentration. Ordinarily, the renal threshold concentration corresponds to a plasma glucose level of 150 mg. per cent or so, and this is only transitorily exceeded after ingestion of a large amount of carbohydrate, during emotional stress, etc. In the rare condition known as renal diabetes the renal reabsorption of glucose is so diminished that significant amounts escape into the urine, and after poisoning with the drug phlorizin the renal reabsorption of glucose is almost completely abolished (*phlorizin diabetes*, see p. 1022). The excretion of blood glucose in the urine in diabetes mellitus is due more to the elevated concentration in the blood than to any significant alteration in the renal threshold. In none of the various conditions described which lead to excretion of glucose in the urine is the loss from the blood ordinarily sufficient to affect appreciably the level of the blood sugar concentration.

Hormonal Control of Carbohydrate Metabolism. The metabolism of carbohydrate in the animal body is under the control of hormones from several of the endocrine glands, of which the pancreas, the anterior pituitary gland, and the adrenal cortex appear to be of major importance. Some aspects of this hormonal control have been indicated in the preceding pages; the endocrine glands themselves are discussed in Chapter 26.

Knowledge concerning the relation between the pancreas and carbohydrate metabolism began with the classical demonstration by Von Mering and Minkowski in 1889 that removal of the pancreas in the dog was followed by development of the symptoms of diabetes mellitus. In 1921 Banting, Best, and Macleod obtained the active principle of the pancreas in this connection, the hormone insulin. Administration of insulin to a normal animal leads to a profound hypoglycemia which may result in convulsions or unconsciousness (insulin shock). In the diabetic, a maintained administration of insulin will completely alleviate the condition. Opinions differ as to the mechanism of insulin action; some feel that insulin accelerates carbohydrate oxidation, while others regard its function as primarily concerned with the synthesis of carbohydrate from noncarbohydrate precursors (gluconeogenesis) or possibly with the conversion of carbohydrate to fat. The significance of insulin in the hexokinase reaction is discussed on the following page.

A further notable advance in knowledge concerning the hormonal control of carbohydrate metabolism was the demonstration by Houssay that removal of the pituitary gland ameliorated the symptoms of diabetes in the pancreatectomized dog. Removal of the adrenal cortex is likewise effective in ameliorating the symptoms of pancreatic diabetes (Long and Lukens). These and other results suggest that control of carbohydrate metabolism by the hormones of the pancreas, the anterior pituitary gland, and the adrenal cortex is essentially a balance between opposing forces, with the effect of insulin opposed by either or both the pituitary and adrenal-cortex principles. Striking confirmation of this concept is afforded by the work of the Coris and their associates, who have found that the ability of tissue extracts to phosphorylate glucose by the hexokinase (glucokinase) reaction (see p. 990) is inhibited by a fraction of anterior-pituitary extract, and also by adrenal-cortical extract; this inhibition is overcome by insulin. Tissue extracts from diabetic animals showed an inhibited hexokinase activity which was overcome *in vitro* or *in vivo* by insulin. This was the first demonstration of the hormonal control of a specific enzymatic reaction of carbohydrate metabolism; these experiments have been confirmed by others.

In another series of experiments, Sutherland and Cori[10] present evidence which indicates that the phosphorylase enzyme of the liver is under the control of epinephrine and the *hyperglycemic factor* (HGF, see p. 769) of the pancreas; small amounts of epinephrine or of HGF markedly increased phosphorylase activity in liver slices where activity was low, and preserved activity in liver homogenates; similar effects were observed *in vivo*. It is suggested that phosphorylase activity in liver represents a balance between inactivation and resynthesis of active enzyme, and that this is in some as yet unknown way under hormonal control. It will be recalled that both epinephrine and HGF promote the breakdown of liver glycogen in the animal body.

Knowledge concerning relationships between the various other hormones of the body and carbohydrate metabolism is relatively meager and difficult to evaluate. The thyroid hormone influences the rate of metabolism in the tissues, but no specific relation to carbohydrate metabolism has been demonstrated, although a role in gluconeogenesis has been postulated. A role in gluconeogenesis has likewise been delegated to certain of the steroid hormones of the adrenal cortex (see Chapter 26). The subject is rendered difficult by the lack of purity of the hormone preparations in some instances, and by the possibility that a postulated action or lack of action may in reality involve the mediation of some other gland or hormone in addition to the one thought to be concerned.

FAT METABOLISM

The fats of the diet and of the animal body represent largely a concentrated form of energy, for metabolic or storage purposes, but there is adequate evidence that fats may subserve important noncaloric metabolic functions as well. In this connection, Burr and Barnes conclude that "there are ample reasons for recommending that the fat intake be not reduced much below the normal established by habit." Thus the fat content of the diet may influence such diverse processes as the digestibility and absorbability of other foodstuffs in the gastrointestinal tract, and the rate of calcification of the bones; fatty acids represent important constituents of the lipide structural components of tissues; the fats of the diet serve as a vehicle for the fat-soluble vitamins; and the presence in the diet of certain highly unsaturated fatty acids which cannot be synthesized by the animal body has been shown to be necessary for normal growth and tissue metabolism. Moreover, the presence of fat in protein-free diets exerts a favorable effect on nitrogen retention, as well as on the course of pregnancy and lactation. The physical capacity of rats has been shown to be greater on high-fat diets than on fat-free regimens. Dietary fat has likewise been shown to exert beneficial effects on rats subjected to such stress factors as thyrotoxicosis,[11] x-radiation,[12] and cold.[13]

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[10] Sutherland and Cori: *J. Biol. Chem.*, **188**, 531 (1951).

The proportion of total calories furnished by dietary fat may vary widely from one group of individuals to another, and depends chiefly on such factors as availability, cost, and established nutritional customs. The recommendation of the National Research Council is that fat be present in the diet to the extent of furnishing 20 to 25 per cent of the total calories (30 to 35 per cent at higher levels of calorie expenditure) and that at least 1 per cent of the total calories be in the form of the essential unsaturated fatty acids (see p. 1004).

Comparative Nutritive Value of Fats. The variety of fats found in nature has stimulated investigation into their relative nutritional merits, for economic as well as for nutritive reasons. These studies have failed to reveal any significant nutritional differences between the common animal and vegetable food fats and oils other than those attributable to slight variation in digestibility or in content of fat-soluble vitamins. Deuel, *et al.*[14] have furnished experimental evidence to refute the concept that butter fat possesses certain saturated fatty acids, not present in other fats, which are essential for growth. While rats prefer a diet containing butter to one in which the fat is corn, cottonseed, olive, peanut, or soybean oil or margarine, this preference is apparently due to flavor alone; margarine fat and butter fat promote similar growth responses when fed to weanling rats under controlled conditions.[15] Deuel has reported that adequate growth and reproduction have been maintained in rats through 36 generations on a diet in which skimmed milk powder and margarine fat replaced the whole milk powder of Sherman's well-known whole milk powder–ground whole wheat basal diet. The equivalence of margarine and butter diets in promoting the growth of children has been demonstrated in tests carried out over a two-year period under well-controlled conditions.[16] These and other results lead to the conclusion that butter fat possesses no special nutritive powers as compared to vegetable fats, and that properly fortified margarine is an adequate substitute for butter and has substantially equivalent nutritional value.[17] Fortification of the first American margarine was carried out in the laboratory of the senior author, who also demonstrated in rats that margarine so fortified was the nutritive equivalent of butter.[18]

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[11] Greenberg and Deuel: *J. Nutrition*, **42**, 279 (1950).

[12] Cheng, Kryder, Bergquist, and Deuel: *J. Nutrition*, **48**, 161 (1952).

[13] Mitchell, Glickman, Lambert, Keeton, and Fahnestock: *Am. J. Physiol.*, **146**, 84 (1946).

[14] Deuel, Movitt, Hallman, and Mattson: *J. Nutrition*, **27**, 107 (1944).

[15] Deuel, Movitt, and Hallman: *Science*, **98**, 139 (1943).

[16] Leichenger, Eisenberg, and Carlson: *J. Am. Med. Assoc.*, **136**, 388 (1948).

[17] See Deuel: *Science*, **103**, 183 (1946); *J. Nutrition*, **32**, 69 (1946); also Editorial, *J. Am. Med. Assoc.*, **128**, 881 (1945).

Studies on the digestibility of natural fats and oils have shown that the common cooking oils such as cottonseed, corn, peanut, and olive oils, as well as butter, lard, and various margarines, are equally (95 per cent) digestible. Beef and mutton tallow are somewhat less digestible. Rapeseed oil, which contains a large proportion of erucic acid, has a digestibility coefficient of only 82 in the rat,[19] although it is almost completely digested in man.[20] Moreover, castor oil does not produce the catharsis in guinea pigs, rabbits, rats, or sheep that it does in human beings. Under such conditions, it is utilized to the extent of 92 to 99 per cent.[21] Among the fatty acids themselves, myristic and lauric acids are practically completely digestible by the rat when fed in olive oil, stearic acid has a low digestibility, and palmitic acid occupies an intermediate position.[22] The triglycerides of these acids behave in the same manner.

Relation Between Diet Fat and Body Fat. Prior to the work of Schoenheimer and Rittenberg on fat metabolism as studied with the aid of fats labeled with isotopic hydrogen (deuterium), it had been believed generally that the fats of the diet were directly metabolized and that only the excess above energy requirements was stored in the fat depots of the body. These authors[23] showed that even when fat is fed at a low level, some is first deposited in the tissues instead of being subjected to direct combustion. The experimental details are of interest. Normal mice were kept on a diet low in fat, and containing 1 per cent of linseed oil which had been partially hydrogenated with deuterium. Later post mortem examination of the body fat for the presence of the deuterio fat indicated that, even though the total amount of depot fat remained constant, 47 per cent of the dietary fat had been incorporated in the depot fat. Thus the animals were burning an approximately equal mixture of depot fat and food fat, in spite of the fact that the fat content of the diet was below that needed for energy purposes if completely burned.

Depot fat is therefore not inert storage material but is constantly involved in metabolic processes. The admixture of dietary and tissue fat shown by such experiments as that just described is, however, subject to some modifying control, since the animal tends to produce a body fat of fairly uniform composition and succeeds moderately well as long as the fat of the diet is not altered to too great an extent. If, however, large amounts of fats containing foreign types of fatty acids are taken in, some of these may be deposited in the tissues unchanged and may modify the character of the body fat. Thus the melting point of the body fat of the dog has been raised from the usual 20° C. to 40° C. by feeding mutto tallow and has been decreased to 0° C. by feeding linseed oil. Anderson and Mendel fed rats diets of skimmed-milk powder and added fat, and found that the iodine number of the body fat could be varied from 122 to 35 by feeding soybean and coconut oils with iodine numbers of 132 and 7.7, respectively. This matter is of commercial importance since feeding hogs rations too high in liquid fats gives rise to a soft body fat yielding a soft lard. In such cases, feeding for some time of a diet high in carbohydrate has a hardening effect, since the fat formed from carbohydrate has a higher melting point and tends gradually to replace a portion of the lower-melting-point fats.

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[18] Hawk: *Am. Food J.*, **19**, 379 (1924).

[19] Deuel, Cheng, and Morehouse: *J. Nutrition*, **35**, 295 (1948).

[20] Holmes: *U.S. Dept. Agric. Bull. No. 687 (1918)*; Deuel, Johnson, Calbert, Gardne and Thomas: *J. Nutrition*, **38**, 369 (1948).

[21] Paul and McCay: *Arch. Biochem.*, **1**, 247 (1942); Stewart and Sinclair: *Arch. Biochem* **8**, 7 (1945).

[22] Hoagland and Snider: *J. Nutrition*, **26**, 219 (1943).

[23] Schoenheimer and Rittenberg: *J. Biol. Chem.*, **111**, 175 (1935).

However, animals vary considerably with regard to the susceptibility of tissue fats to alteration in composition due to the fats present in the diet. Shorland[24] designates the body fats from species such as cows, sheep, and other ruminants which resist change due to diet as *homolipides*, while the fats from species which respond more or less readily to food fats of unusual composition by a change in body fat composition are called *heterolipides*. In considering this problem from an evolutionary viewpoint, it has been suggested that the storage depot fats of the lower marine forms, which have the most complex fatty acid make-up of all species, merely mirror the composition of the food fats.[25] This is because such animals are unable to dilute the food fat with synthetic fats, since they do not possess the ability to change carbohydrate and protein into fat. As the capacity for transforming these nonlipide components into fatty acids develops in the forms ranking higher in the evolutionary pattern, the modification of the storage fat by diet becomes less pronounced. In the ruminants, most of the storage fat is newly synthesized from protein and carbohydrate; dietary fat is largely modified in the complicated gastrointestinal tract, and it exerts no appreciable effect upon the composition of the fat in the storage depots. According to Wertheimer and Shapiro,[26] synthesis of fat from carbohydrate actually takes place in the adipose tissue.

Some modification of ingested fat may occur during absorption and resynthesis in the intestinal wall. That the various saturated fatty acids found in the mixed triglycerides of animal body fats are in synthetic equilibrium is shown by the fact that, after the feeding of a particular fatty acid labeled with deuterium i.e., deuterio-palmitic acid, a significant amount of the deuterium is subsequently found incorporated in the other saturated fatty acids of the body fats. Deuterium is also found in the oleic and palmitoleic acid fractions, thus proving that these mono-unsaturated acids may be synthesized from dietary components.[27] This synthesis may be by direct dehydrogenation, but it appears more probable that the fed fatty acid enters into degradation reactions to produce smaller fragments from which the oleic acid is then synthesized. No deuterium is found in the linoleic acid fraction, thus confirming the original observations of Burr and Burr (see p. 1004) that the animal is unable to synthesize these highly unsaturated fatty acids.

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[24] Shorland: *Nature*, **165**, 766 (1950).

[25] Shorland: *Nature*, **170**, 924 (1952).

[26] Wertheimer and Shapiro: *Physiol. Revs.*, **28**, 451 (1948).

[27] Stetten and Schoenheimer: *J. Biol. Chem.*, **133**, 329 (1940).

Essential Fatty Acids. The inability of the animal body to synthesize certain highly unsaturated fatty acids essential for its normal nutrition was first demonstrated by Burr and Burr,[28] in 1929. These investigators found that rats placed on a diet devoid of fat but otherwise apparently complete failed to grow and developed characteristic lesions of the skin and tail (see p. 1073 and Fig. 268 for complete description of this condition). Addition to the diet of the normal saturated fatty acids or of oleic acid did not render the diet complete; in fact, the administration of hydrogenated coconut oil has been shown to shorten the depletion period of rats on a diet free from essential fatty acids, as well as to intensify the accompanying symptoms.[29] When triolein was the fat employed, this effect was not noted.[30] When small amounts of highly unsaturated fatty acids such as linoleic or linolenic acid were added to the diet the deficiency did not develop. Further study has confirmed these findings; the deficiency is apparently not concerned with the formation of fat from carbohydrate, or fat storage; arachidonic acid is more effective than the other unsaturated fatty acids in promoting growth; and in the deficient animal the arachidonic acid content of the liver is maintained at the expense of continuous depletion of the other tissues.[31] This would imply that the presence of highly unsaturated fatty acids in liver, at one time thought to be a characteristic of fat metabolism in this organ, is in reality due to selection and retention of these compounds from the blood. Arachidonic acid appears to be the essential fatty acid which is the physiological member of the group. Using spectrophotometric procedures for the determination of the number of double bonds in the fatty acid fraction of blood and tissue fats, Reiser[32] and Witten and Holman[33] have demonstrated the synthesis of tetraenoic acid (arachidonic) from linoleic acid.

Oxidation of Fats. The glycerol portion of the fat molecule is undoubtedly oxidized in the body by pathways of carbohydrate metabolism, probably after preliminary phosphorylation to form phosphoglycerol, which then by oxidation could give rise to either phosphoglyceric aldehyde or phosphoglyceric acid, both of which are recognized intermediates in carbohydrate breakdown (see p. 989). The fatty acids are oxidized in quite a different way.

β-OXIDATION. It is generally agreed that the major method whereby fatty acids are oxidized in the animal body is by the process known as β-oxidation. According to this concept, the fatty acid chain is oxidized at the carbon atom β to the carboxyl group, with the splitting off of a

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[28] Burr and Burr: *J. Biol. Chem.*, **82**, 345 (1929); **86**, 587 (1930).

[29] Evans and Lepkovsky: *J. Biol. Chem.*, **96**, 157 (1932); Deuel, Greenberg, Anisfeld, and Melnick: *J. Nutrition*, **45**, 535 (1951).

[30] Alfin-Slater, Bingemann, and Deuel: Unpublished observations (1953).

[31] Smedley-MacLean and Nunn: *Biochem. J.*, **35**, 983 (1941); Smedley-MacLean and Hume: *Biochem. J.*, **35**, 990, 996 (1941).

[32] Reiser: *J. Nutrition*, **42**, 325 (1950).

[33] Witten and Holman: *Arch. Biochem. Biophys.*, **37**, 90 (1952).

2-carbon fragment and the production of a fatty acid having two less carbon atoms than the original. This process continues along the chain until the entire fatty acid molecule has been broken down to 2-carbon fragments by the removal of two carbon atoms at a time.

The first biological evidence for β-oxidation was afforded by the experiments of Knoop. This investigator found that on feeding animals fatty acids of varying chain length but in each case with the ω-carbon (the one farthest removed from the carboxyl carbon) attached to a phenyl group, there could be isolated from the urine either benzoic acid or phenylacetic acid, in the form of the conjugated derivatives hippuric acid and phenaceturic acid, respectively. Benzoic acid was obtained when the fatty acid chain contained three or five carbon atoms; phenylacetic acid when it contained two (i.e., phenylacetic acid itself) or four carbon atoms. These results led Knoop to conclude that the carbon atoms could not be removed one by one during oxidative breakdown of the fatty acid, but must come off in pairs, i.e., by oxidative removal at the β-carbon.

The mechanism of β-oxidation, and the nature and fate of the 2-carbon fragment thereby produced, has been the subject of prolonged and intensive study. A major difficulty in the past, since overcome, has been the inability to obtain cell-free preparations from animal tissues which were capable of oxidizing fatty acids and were susceptible to experimental study. Munoz and Leloir[34] and Lehninger[35] were the first to describe such preparations from liver tissue. Lehninger's preparations under the proper conditions readily brought about the oxidation of all of the normal saturated fatty acids containing from 4 to 16 carbon atoms, with acetoacetic acid as the main product. In the oxidation of octanoic acid, for example, two moles of acetoacetic acid could be obtained for each mole of octanoic acid oxidized:

$$\underset{\textbf{Octanoic acid}}{CH_3(CH_2)_6COOH} + 2O_2 \rightarrow \underset{\textbf{Acetoacetic acid}}{2CH_3{\cdot}CO{\cdot}CH_2{\cdot}COOH}$$

The interesting observation was made that if oxalacetate was added to the system, the yield of acetoacetate decreased and some citrate, α-ketoglutarate, and succinate were formed, suggesting that the citric acid cycle of Krebs (see p. 990) was entering into the picture. Since added acetoacetate was not oxidized, even in the presence of oxalacetate, and pyruvate was found to be oxidized in a manner similar to the oxidation of fatty acids, it was postulated that both fatty acid oxidation and pyruvate oxidation produced a reactive 2-carbon fragment as a common intermediate. This 2-carbon compound could then condense either with itself to form acetoacetate as end product, or with oxalacetate to form citrate, the citrate then entering the oxidative metabolism of the Krebs cycle. With the identification of the reactive 2-carbon fragment as the acetyl group of acetyl-coenzyme A (p. 1010), the process is now pictured by Lehninger as follows:

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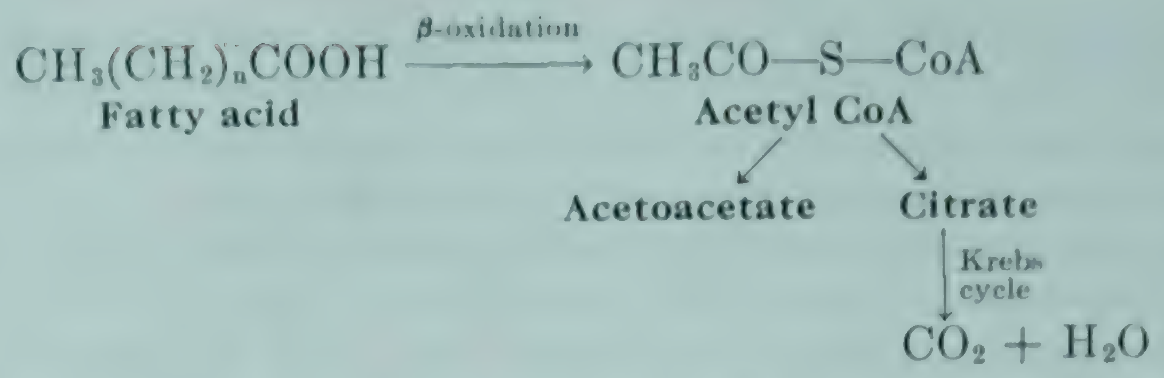
[34] Munoz and Leloir: *J. Biol. Chem.*, **147**, 355 (1943).
[35] Lehninger: *J. Biol. Chem.*, **161**, 437 (1945).



Further aspects of the metabolic significance of acetoacetic acid and acetyl groups are considered on pp. 1007 and 1010. In connection with the breakdown of long-chain fatty acids to the stage of acetyl groups, it has been suggested that the first step in the reaction is the formation of a fatty acid–coenzyme A combination, through the mediation of adenosinetriphosphate (ATP). In the case of heart-muscle preparations which oxidize fatty acids, Mahler[36] has formulated this reaction (for butyrate) as follows:

$$\text{Butyrate} + \text{ATP} + \text{CoA} \rightarrow \text{Butyryl CoA} + \text{Pyrophosphate} + \text{AMP}$$

where AMP is adenosinemonophosphate, or adenylic acid. Presumably a similar reaction occurs with other fatty acids as well. Evidence to this effect has been obtained by the use of hydroxylamine to "trap" fatty acid esters of coenzyme A through the formation and identification of the corresponding hydroxamic acids:

$$\underset{\textbf{Acyl coenzyme A}}{\text{R—CO—S—CoA}} + \underset{\textbf{Hydroxylamine}}{H_2NOH} \rightarrow \underset{\textbf{Acyl hydroxamic acid}}{\text{R—CO—NHOH}} + \underset{\textbf{Coenzyme A}}{\text{HS—CoA}}$$

Using this procedure, caprylyl hydroxamic acid has been isolated from liver extracts actively oxidizing caprylic acid, the oxidation presumably going through the intermediate formation of a caprylyl–coenzyme A ester.

A second method for the formation of fatty acid–coenzyme A combinations, which thus far appears limited in its applicability to butyric and acetoacetic acids, is through exchange with succinyl CoA. According to Mahler,[36] oxidation of α-ketoglutaric acid to succinic acid in the presence of coenzyme A gives rise to the intermediate formation of succinyl CoA, which can then convert butyric acid into butyryl CoA:

$$\text{Succinyl CoA} + \text{Butyrate} \rightarrow \text{Butyryl CoA} + \text{Succinate}$$

It is to be noted that this reaction involves DPN (in the ketoglutarate-succinate stage) but not ATP, and it may account for the differences which have been noted occasionally between the oxidative metabolism of butyric acid and that of other fatty acids.

It appears likely that the fatty acid–coenzyme A combination, once formed, is a substrate for a dehydrogenase action involving DPN and resulting in the formation of a β-keto group in the fatty acid chain:

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[36] Mahler: *Phosphorus Metabolism*, Vol. II, Baltimore, Johns Hopkins University Press, 1952.

$$RCH_2CH_2CO—S—CoA + 2DPN \rightarrow RCOCH_2CO—S—CoA + 2DPNH + 2H^+$$

According to current views, the β-keto acyl ester thus formed can react with another molecule of coenzyme A at the keto group, to split off acetyl CoA and leave an acyl–coenzyme A combination containing two less carbon atoms:

$$RCOCH_2CO—S—CoA + HS—CoA \rightarrow RCO—S—CoA + CH_3CO—S—CoA$$

Thus by this thioclastic reaction the acetoacetyl CoA formed in butyrate oxidation will give rise to acetyl CoA, whereas for the higher fatty acids the combination of dehydrogenase activity and reaction with coenzyme A results in the shortening of the chain by 2 carbon atoms at a time, with the concomitant release of acetyl groups in the form of acetyl CoA, until the entire molecule is broken down.

As stated above, the acetyl groups which are the intermediate products of fatty acid oxidation give rise to acetoacetic acid or are further oxidized by the metabolic processes of the Krebs cycle. There is some evidence that acetyl groups derived from the carboxyl end of a fatty acid chain may have a somewhat different metabolic significance from those derived from the methyl end of the chain; further work will be needed to clarify this point. There is much in the picture of fatty acid oxidation presented here that is obscure and that will presumably be subject to modification as knowledge increases in this field. At the same time, it is believed that a reasonably satisfactory explanation is now available for most of the known facts regarding fatty acid oxidation in animal tissues, and that this explanation represents a significant advance in understanding over previous concepts. It is likely that other pathways of fat oxidation exist. For example, it is known that fatty acids may undergo ω-oxidation—i.e., oxidation beginning at the carbon atom farthest removed from the carboxyl group—since certain long-chain dicarboxylic acids have been isolated from the urine of animals after the feeding of fatty acids. The significance, if any, of this type of oxidation in the normal metabolism of fatty acids is obscure.

Metabolism of Acetoacetic Acid. Acetoacetic acid, its equilibrium reduction product β-hydroxybutyric acid, $CH_3 \cdot CHOH \cdot CH_2 \cdot COOH$, and its decarboxylation product acetone, $CH_3 \cdot CO \cdot CH_3$, are the ketone bodies ("acetone bodies") which normally are found only in very small amounts in the blood and urine. Under certain conditions, as in diabetes mellitus, during starvation, or prolonged subsistence on a low-carbohydrate diet, the amount of these ketone bodies in the blood rises and considerable quantities may be excreted in the urine (see experiment on p. 1073). Such a condition is known as ketosis. It was believed for some time that acetoacetic acid and its associated ketone bodies were abnormal metabolic end products, and in particular that acetoacetic acid represented the inability of the tissues to carry fatty acid oxidation by the β-oxidation process beyond the stage of the 4-carbon compound.

It is now known that acetoacetic acid is a normal end product of fatty acid oxidation in liver. The acetoacetic acid formed in liver is not further utilized by this organ, except possibly to a slight extent in fasting. Other tissues, however, readily metabolize acetoacetic acid to CO_2 and H_2O, and there appears to be no impairment in this respect in diabetes (Soskin). The intensity of ketone-body production (ketogenesis) by liver appears to be largely a question of substrate availability. If adequate carbohydrate is available, the liver apparently prefers carbohydrate oxidation as a source of energy, and ketone-body production is small. Carbohydrate is therefore an "antiketogenic" substance. In the absence of carbohydrate oxidation, as in diabetes or when glycogen stores are exhausted, oxidative energy is derived almost entirely from fatty acid breakdown and ketone bodies result. They may be produced by the liver in such quantities that the peripheral tissues are unable to oxidize them as fast as they are formed, in which case they will accumulate in the blood (ketonemia) and be excreted in the urine (ketonuria).

It was felt at one time that ketogenesis was harmful and that it could be controlled by the proper ratio in the diet of ketogenic material (fats, and the ketogenic portion of proteins) to antiketogenic material (carbohydrate, and the glucogenic portion of protein). These views are no longer held. The major effect of ketosis on the animal body appears to be in relation to acid-base balance; excretion of large amounts of acetoacetic acid and β-hydroxybutyric acid in the urine as their alkali salts depletes the body of available base and may lead to the development of an acidosis.

That ketone bodies may arise from sources other than fatty acid oxidation is well recognized. Their origin from pyruvate under certain circumstances has been shown, and it is well established that certain amino acids such as leucine and phenylalanine are metabolized via the intermediate formation of acetoacetic acid. Presumably any metabolic source of acetic acid could also serve as a source of acetoacetic acid (see p. 1009). Thus ketone body production is not a characteristic of fatty acid oxidation alone, but is rather to be considered only one of the various metabolic processes which yield energy to the organism.

Ketonuria during fasting occurs spontaneously to an appreciable extent only in man and the higher apes. However, when the salts of ketogenic acids are administered to fasting rats, a marked ketonuria obtains, which is physiologic. This is defined as an *exogenous* ketonuria. Likewise, after the development of fatty livers in rats, a spontaneous ketonuria of considerable magnitude results on fasting; this type of ketonuria is known as *endogenous* ketonuria. Women exhibit a much higher degree of fasting ketonuria than do men;[37] the same sex difference has been shown to occur in both the exogenous[38] and the endogenous types[39] of ketonuria in the rat.

Further stages in the breakdown of acetoacetic acid to CO_2 and H_2O occur largely in tissues other than the liver and are considered to involve the participation of coenzyme A and the reaction mechanism of the Krebs tricarboxylic acid cycle. The role of the Krebs cycle in acetoacetate metabolism was first postulated by Breusch[40] and by Wieland[41] and has been confirmed by other investigators. For example, Buchanan *et al.*[42] found that, after incubating kidney tissue with carbon-labeled acetoacetate in the presence of an excess of nonisotopic ketoglutarate, succinate, and fumarate, on subsequent isolation these three intermediates in the Krebs cycle contained isotopic carbon derived from the acetoacetate. These results, and those of many other investigators, leave no doubt concerning the participation of the Krebs cycle in acetoacetate metabolism.

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[37] Deuel and Gulick: *J. Biol. Chem.*, **96**, 25 (1932).

[38] Butts and Deuel: *J. Biol. Chem.*, **100**, 415 (1933).

[39] Deuel, Hallman, and Murray: *J. Biol. Chem.*, **119**, 257 (1937).

[40] Breusch: *Science*, **97**, 490 (1943).

[41] Wieland and Rosenthal: *Ann.*, **554**, 241 (1943).

In the discussion of the β-oxidation of fatty acids on p. 1005, it was pointed out that the production of acetoacetate itself as an intermediate in the breakdown of fatty acids by extrahepatic tissue is considered to be unlikely; rather, there is formed the acetoacetyl ester of coenzyme A, acetoacetyl CoA. This β-keto acyl ester can undergo the thioclastic reaction with more coenzyme A described on p. 1007, to give rise to acetyl CoA, which then follows well-recognized metabolic pathways discussed below. If this view is correct, then acetoacetic acid formed by the liver and brought by the blood to the extrahepatic tissues must be converted into acetoacetyl CoA before being further metabolized. One mechanism for the activation of acetoacetic acid is found in the reactions described by Mahler[36] and discussed earlier in this chapter; according to this report, acetoacetate is similar to butyrate in its ability to react with succinyl CoA to give (presumably) acetoacetyl CoA and succinate. If this or an equivalent reaction is found to prevail generally in the extrahepatic tissues, the metabolism of acetoacetate by these tissues becomes readily understood.

The possible relationship between fat and carbohydrate metabolism exemplified by the entrance of the reactions of the tricarboxylic acid cycle into acetoacetate metabolism may afford some basis for the aphorism that "fats burn in the fire of the carbohydrates," which has fallen into some disrepute in recent years, possibly because it is clearly not applicable to fat metabolism in the liver. Certainly if it can be shown that carbohydrate metabolism facilitates acetoacetate combustion (which has not as yet been done but which appears likely) the existence of a "ketolytic" as well as an antiketogenic action of carbohydrate must once again be considered, at least in reference to organs other than liver. For further discussions of ketone bodies, see Chapters 29 and 31.

Metabolism of Acetic Acid. Acetic acid is the simplest possible fatty acid with an even number of carbon atoms. It is readily metabolizable when fed to an animal, or when incubated with liver, kidney, or heart tissue (but not brain or muscle). It has been somewhat difficult to evaluate the metabolic significance of acetic acid in the past, because of its metabolic lability and the lack of precise methods for its quantitative determination. By the use of isotope labeling, acetic acid has been implicated in the synthesis of a variety of substances such as acetoacetic acid, fatty acids, cholesterol, protoporphyrin, glycogen, and the dicarboxylic amino acids, as well as in the acetylation of choline to form acetylcholine and of such substances as sulfanilamide and *p*-aminobenzoic acid, which are excreted in part as acetyl derivatives in the urine. On the basis of the "dilution" of isotopic dietary acetate by the nonisotopic acetate of the tissues,

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[42] Buchanan, Sakami, Gurin, and Wilson: *J. Biol. Chem.*, **159**, 695 (1945).

Bloch and Rittenberg[43] estimate that a 100-g. rat produces about 1 g. of acetic acid per day by all metabolic processes, of which both carbohydrate breakdown and the β-oxidation of fatty acids are major components. Since the animal normally excretes little if any acetic acid in the urine, all of the acetic acid produced must be further metabolized.

The mechanisms of acetate metabolism have been intensively studied in many laboratories. As is evident from the previous discussions in this chapter, the oxidative breakdown of both carbohydrate and fatty acids appeared to require the formation of an intermediate 2-carbon compound which was clearly not acetate itself but which was closely similar to acetate, and hence called by many *active acetate*. Studies on the generation of acetyl groups by animal tissues, e.g., the formation of acetylsulfanilamide from sulfanilamide by liver tissue, likewise pointed to the existence of an active form of acetate. A major contribution to this field was made by Lipmann in the discovery of coenzyme A (for acetate) as the coenzyme required in the enzymatic transformations of acetate by bacterial and animal tissues. Many illustrations of the role of coenzyme A in carbohydrate and fat oxidation have already been presented in this chapter; the structure and vitamin relationships of coenzyme A will be found in Chapter 35.

The search for active acetate appears to have reached a successful conclusion in the discovery by Lynen[44] of the formation of the S-acetyl ester of coenzyme A as an intermediate in the metabolism of acetate by yeast. Coenzyme A contains a mercaptoethanolamine residue in the molecule; the relation between the structures of coenzyme A and acetyl coenzyme A is as follows:

$R{-}NH{-}CH_2{-}CH_2SH$
Coenzyme A

$R{-}NH{-}CH_2{-}CH_2{-}S{-}CO{-}CH_3$
Acetyl Coenzyme A

Acetyl coenzyme A fulfills all of the requirements for active acetate in the test systems thus far studied. For example, while ordinary acetate requires the presence of ATP to form citrate in tissue extracts containing oxalacetate, acetyl CoA yields citrate without ATP being present. Similarly, the requirement for a source of energy in the formation of acetylcholine and acetylsulfanilamide by tissue preparations disappears if acetyl CoA rather than acetate is used as a source of acetyl groups. Other examples of the significance of acetyl CoA in reactions requiring the presence of active acetate have been given in the discussions of carbohydrate and fat oxidation.

It is clear therefore that acetate as such is essentially inert metabolically until it is activated by conversion to acetyl CoA. That such activation occurs in animal tissues is generally believed; the mechanism however is still not clear. Lipmann[45] has shown in both yeast and liver extracts that the formation of acetyl CoA from acetate in the presence of ATP may occur as follows:

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[43] Bloch and Rittenberg: *J. Biol. Chem.*, **159**, 45 (1945).

[44] Lynen and Reichert: *Angew. Chem.*, **63**, 47 (1951).

[45] Lipmann, Jones, Black and Flynn: *J. Am. Chem. Soc.*, **74**, 2384 (1952).

Coenzyme A + ATP → Coenzyme A pyrophosphate + Adenylic acid

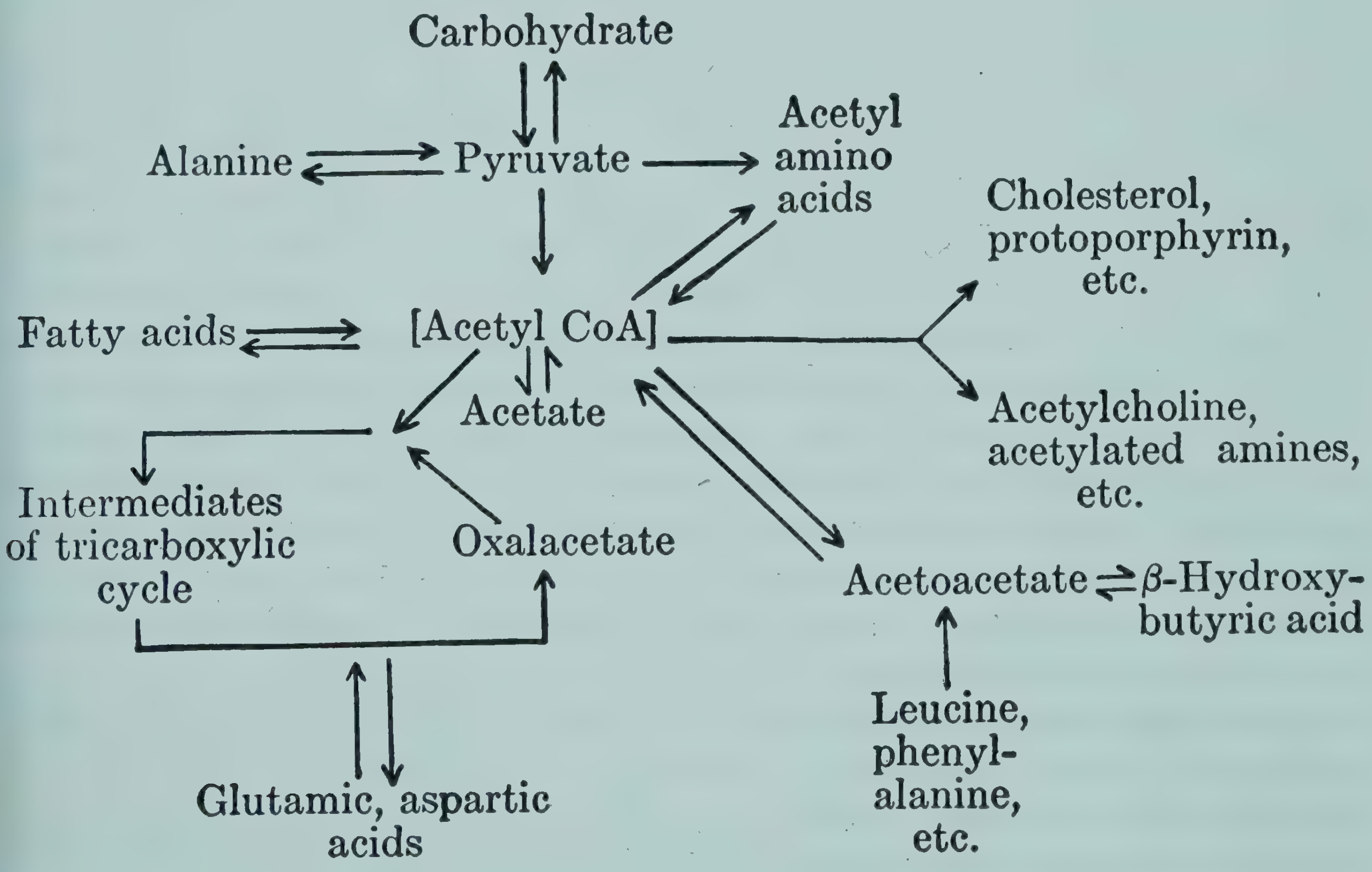
Coenzyme A pyrophosphate + Acetate → Acetyl CoA + Pyrophosphate

Another reaction found in bacteria which is of possible interest here involves the transacetylation of acetyl groups from acetylphosphate to coenzyme A:

Acetylphosphate + CoA ⇄ Acetyl CoA + Phosphate

Acetylphosphoric acid has the structure: $CH_3—CO—OPO_3H_2$. Its role in bacteria as a precursor of acetyl CoA (and hence of acetate) is as indicated in the above equation; the precise significance of acetylphosphate in animal tissues is not clearly defined. The presence of a transacetylase has not been demonstrated in animal tissue extracts; if bacterial transacetylase is added to such extracts, acetylphosphate is readily utilized as a source of active acetate through the intermediate formation of acetyl CoA.

Available evidence concerning the metabolic pathways of acetic acid is summarized in the following diagram:


Carbohydrate
Alanine
Pyruvate
Acetyl amino acids
Cholesterol, protoporphyrin, etc.
Fatty acids
[Acetyl CoA]
Acetate
Acetylcholine, acetylated amines, etc.
Intermediates of tricarboxylic cycle
Oxalacetate
Acetoacetate
β-Hydroxy-butyric acid
Leucine, phenyl-alanine, etc.
Glutamic, aspartic acids

It will be noted that, according to this formulation, acetic acid in the form of acetyl CoA occupies a key position in the metabolism of not only fatty acids but also carbohydrate and certain amino acids; in fact, the diagram may be taken as a general summary of the metabolic interrelationships of carbohydrate, fat, and protein.

The participation of acetate in the formation of such compounds as acetylcholine and acetylsulfanilamide has been definitely established; both reactions require acetyl CoA although taking place in different tissues (brain and liver, respectively).

The formation of acetyl derivatives of foreign aromatic amines (sulfanilamide, *p*-aminobenzoic acid) and amino acids (phenylaminobutyric acid) has been studied with the aid of the various available isotopes, and by other means; both acetic acid and pyruvic acid have been shown to be precursors of acetyl groups in this connection, with possible qualitative and quantitative differences between them. The significance of the origin of acetyl amino acids from pyruvate is further discussed on p. 1018, in connection with amino acid metabolism.

Interconvertibility of Fat and Carbohydrate. That the fat of the body may arise from carbohydrate has long been known (see p. 996). The reverse process, the production of carbohydrate from fat and particularly from the fatty acid portion, has been the subject of considerable controversy. Experimental methods based upon the production of extra glucose or glycogen by which it is readily possible to demonstrate the glucogenic power of certain amino acids, for example, yield essentially negative results when fatty acids are studied. For these and other reasons, many have maintained that fats do not give rise to carbohydrate in the animal body, although this conversion can be demonstrated in certain other organisms. The opposing view, held by some, is that gluconeogenesis from fatty acids is readily possible and indeed is the major explanation for the hyperglycemia of diabetes mellitus. Much of the evidence cited in favor of this latter view is faulty. It has nevertheless been unequivocally demonstrated by the use of isotopes that the carbon of certain fatty acids can be incorporated into the glycogen of the animal body,[46] thus proving that fatty acids and carbohydrate are reversibly related, as would indeed be expected from the equilibrium relations shown on p. 1011. The amount of conversion found, however, is extremely small relative to that expected if the conversion of fatty acid to carbohydrate were of any value to the organism. It has been suggested that such conversion must be uneconomical to the body in the light of current concepts of carbohydrate metabolism, since carbohydrate formation from fatty acids must inevitably be at the expense of the metabolic energy of carbohydrate breakdown. Whether or not the altered metabolic state of the diabetic facilitates or even requires the conversion of fatty acid to carbohydrate still remains to be unequivocally demonstrated.

Lipotropic Factors. On diets which are high in fats containing much saturated fatty acid and which are low in protein or in choline, there is observed a large increase in the fat content of the liver. A high cholesterol diet likewise leads to the production of a fatty liver, the lipides in this case consisting of cholesterol as well as neutral fat. In either case, an increase in choline in the diet brings about a reduction in liver fat. This is of interest in connection with the possible role of lecithin in fat metabolism, but the explanation for the so-called lipotropic action of choline is not yet established. Methionine likewise exerts a marked lipotropic action, owing to its ability to promote the synthesis of choline by the transfer of methyl groups to suitable precursors (see p. 1024).

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[46] Buchanan, Hastings, and Nesbett: *J. Biol. Chem.*, **150**, 413 (1943); Lorber, Lifson, and Wood. *J. Biol. Chem.*, **161**, 411 (1945).

Other substances which exert a lipotropic action include *lipocaic*, a substance of as yet unknown nature claimed to be present in the pancreas; inositol, a member of the vitamin B complex (Chapter 35) and a constituent of certain phospholipides (Chapter 11); tryptophan, and possibly glutamic acid. Whether these various substances act in an independent fashion or through the mediation of choline action is not clear. Fatty livers are also found under certain conditions in animals deficient in the essential fatty acids, in pantothenic acid or in riboflavin, or in animals which have been provided with an excess of thiamine or biotin (Chapter 35). The relation between these various dietary constituents and the deposition of liver fat is not well defined; it is felt by some that they may represent nonspecific factors acting through some general change in the nutritional state.

Parenteral Fat Administration. Although glucose and amino acids have been employed successfully in intravenous therapy for a number of years, practical procedures for the administration of fat are still in the process of development. The two obstacles which prevented the earlier application of the procedure to man were the difficulty of obtaining a preparation stable enough to resist sterilization and which would not cause fat embolism. However, as early as 1935, in this country, Holt and associates,[47] employing a butter emulsion stabilized with purified commercial egg lecithin and containing 7 to 7.5 per cent of lipides, administered the preparation successfully to infants. The emulsion was prepared by homogenization at 4000 pounds pressure, followed by sterilization; most of the particles were less than 60 μ in diameter. Since this earlier work, a number of satisfactory fat emulsions have been prepared, using butterfat, corn oil, coconut oil, or other fats, by employing soya phosphatide,[48] or glycerol monostearate[49] as a stabilizer, or by subjecting the fat to supersonic radiations.[50] Shafiroff et al.[51] reported that, when fat was administered subcutaneously, a better utilization obtained when the spreading agent *hyaluronidase* was incorporated in the emulsion.

Proof that emulsified fats are utilized following their intravenous administration has been obtained by the use of tagged compounds.[52] For example, Lerner and co-workers have found that, when a C^{14}-labeled palmitic acid was injected intravenously into rats as a tripalmitin emulsion, as much as 59 per cent of the administered isotopic carbon was expired as CO_2 within 24 hours; moreover, considerable amounts of the injected fatty acid were stored in the several adipose tissues. The bulk of the C^{14}-labeled fatty acid stored in the liver and intestine was present in the phospholipide fraction. Further confirmation that fat can be used when injected intravenously has been adduced by Mann *et al.*,[53] who proved that it spares protein in growing puppies, and provides energy; as much as 30 per cent of the total energy requirement could be used efficiently in the form of intravenous fat.

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[47] Holt, Tidwell, and Scott: *J. Pediat.*, **6**, 151 (1935).

[48] Collins, Kraft, Kinney, Davison, Young, and Stare: *J. Lab. Clin. Med.*, **33**, 143 (1948); Mann, Geyer, Watkin, and Stare: *J. Lab. Clin. Med.*, **34**, 699 (1949); Shafiroff, Mulholland, Roth, and Baron: *Proc. Soc. Exp. Biol. Med.*, **70**, 343 (1949).

[49] Lerner, Chaikoff, and Entenman: *Proc. Soc. Exp. Biol. Med.*, **70**, 388 (1949).

[50] Myers and Blumberg: *Proc. Soc. Exp. Biol. Med.*, **35**, 79 (1936).

[51] Shafiroff, Baron, Recht, and Mulholland: *Proc. Soc. Exp. Biol. Med.*, **77**, 608 (1951).

[52] Rutenburg, Seligman, and Fine: *J. Clin. Invest.*, **28**, 1110 (1949); Lerner, Chaikoff, Entenman, and Dauben: *Proc. Soc. Exp. Biol. Med.*, **70**, 384 (1949); Shafiroff, Mulholland, and Baker: *Exp. Med. and Surg.*, **9**, 180 (1951).

Glycerides of short-chain acids were reported to be toxic, while those of lauric, myristic, palmitic, and stearic acids were found to be nontoxic when injected intravenously as emulsions in man. Oleic and linoleic acid esters can be tolerated by human subjects, at least in small concentrations.[54] Geyer and associates[55] reported a moderate increase in the total lipides in the spleen, lungs, and liver when stable emulsions of fats were administered by vein to rats. Gorens *et al.*[56] successfully administered as much as 3 g. of fat daily per kilogram of body weight to adults, and 6 g. of fat per kilogram of body weight to a 7-week-old infant for periods up to 27 days. Under these conditions weight loss was prevented, and positive nitrogen and potassium balances were maintained. Furthermore, fat injections produced no gross or microscopic changes in the tissues. However, one should not overlook the report that intravenous fat produces a febrile response, which is termed *thermogenic* rather than *pyrogenic*, since it is not of bacterial origin.[57] Lambert and associates[57] suggest that this effect may possibly result from an overburdening of the fat storage facilities due to the rapid influx of this foodstuff into the tissues. Considerable work is in progress to find methods of preparation of fat emulsions which will not produce the febrile reaction.

PROTEIN METABOLISM

The proteins of the diet are considered to be completely broken down to their constituent amino acids in the digestive tract by the action of the various proteolytic enzymes present, and to be absorbed into the animal body in the form of these individual amino acids. The requirement for protein is therefore fundamentally (if perhaps not exclusively) a requirement for amino acids, and it should be possible to express the protein requirement of an animal in terms of the amount and kind of amino acids rather than of protein itself. The ability of amino acids to replace protein in the animal diet was first demonstrated many years ago by the use of hydrolyzed protein, supplemented with those amino acids known to be lost during the hydrolysis, in place of protein itself to supply the nitrogen requirements of the animal. This demonstration reaches its peak in the experimental procedure available today, for both human and animal experiments, whereby mixtures of highly purified amino acids, in many cases synthetic products, are used to replace protein completely in experimental diets. The availability of this type of experimental procedure, which was achieved only after the isolation and characterization of the amino acid threonine by McCoy, Meyer, and Rose in 1935, has led to major advances in the science of nutrition. It is also largely responsible for directing attention to the clinical practicality of supplying nitrogen requirements during disease and convalescence by either feeding concentrated intact proteins or intravenous administration of hydrolyzates and amino acid mixtures, an application of nutritional science which promises to be of outstanding medical value. It is an interesting commentary on scientific progress that approximately four decades elapsed between the demonstration that protein hydrolyzates were of nutritional value and the extensive application of this fact in medical practice.

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[53] Mann, Geyer, Watkin, Smythe, Dju, Zamcheck, and Stare: *J. Lab. Clin. Med.*, **33**, 1503 (1948).

[54] Shafiroff, Mulholland, and Baron: *Proc. Soc. Exp. Biol. Med.*, **79**, 721 (1952).

[55] Geyer, Watkin, Matthews, and Stare: *Proc. Soc. Exp. Biol. Med.*, **77**, 872 (1951).

[56] Gorens, Geyer, Matthews, and Stare: *J. Lab. Clin. Med.*, **34**, 1627 (1949).

[57] Lambert, Miller, and Frost: *Am. J. Physiol.*, **164**, 490 (1951).

Dispensable and Indispensable Amino Acids. The amino acids supplied by the protein of the diet are needed for the synthesis of new body proteins during growth and for the continuous regeneration of the tissue proteins of the adult, as well as for many special purposes such as the formation of hormones, enzymes, purines, bile salts, creatine, and many other similar compounds. It has long been recognized that certain of the amino acids needed by the animal body for these various purposes need not necessarily be present in the proteins of the diet, since they can be synthesized within the tissues from suitable precursors. On the other hand, there are certain amino acids required by the body which cannot be synthesized under ordinary circumstances and which therefore *must* be present in the diet in adequate amount or nutritional failure, leading ultimately to death, will result.

Those amino acids which can be synthesized by the body from the other constituents of the ordinary diet at a rate adequate for nutritional demands are called *dispensable* or *nonessential* amino acids; those which cannot be so synthesized but must be present in the diet are called *indispensable* or *essential* amino acids. It must be clearly understood that these terms refer solely to the presence of these amino acids *in the diet;* as far as we know, *all* of the amino acid constituents of the protein molecule are essential in one way or another for the metabolic processes of the animal body. In fact, as du Vigneaud has pointed out, the so-called nonessential amino acids are more properly regarded as being so essential to the animal economy that it has been forced to retain the ability to synthesize them even at the expense of other constituents of the diet.

Knowledge concerning the dietary dispensability or indispensability of various amino acids has been obtained in the past largely by feeding experiments with young animals (e.g., rats) using a diet containing protein known to be low or lacking in certain specific amino acids, or containing a protein hydrolyzate from which certain amino acids (e.g., tyrosine, tryptophan) could be removed. Failure of the animal to grow on such a diet, followed by good growth when the diet was supplemented by the missing amino acids, afforded evidence as to the indispensability of the amino acids in question.

This procedure has certain obvious limitations and occasionally gave obscure results. A more adequate experimental basis is afforded by the use of mixtures of pure amino acids as the sole source of dietary nitrogen (Rose). To establish the nutritional significance of any one amino acid, it is only necessary to prepare the mixture without that particular amino acid and to use this deficient mixture as the source of nitrogen for the animal. Much progress has been made in this field since this experimental technique became available.

As a result of the extensive experiments of Osborne and Mendel, of Hopkins, and of Rose, using the various procedures just described, the following tentative classification of the common amino acids with regard to their dietary dispensability or indispensability for the young growing mammal was suggested by Block:

Indispensable	*Partly Indispensable*		*Dispensable*
	Group A	*Group B*	
Histidine	Cystine	Arginine	Glutamic acid
Lysine	Tyrosine	Glycine	Aspartic acid
Tryptophan			Alanine
Phenylalanine			Serine
Methionine			Proline
Threonine			Hydroxyproline
Leucine			
Isoleucine			
Valine			

As one might expect, the division between the various groups of amino acids is not entirely a sharp one. Thus, although methionine in sufficient amount will supply the needs of the animal for cystine, cystine is an important constituent of the diet in that it is able to spare that portion of methionine which otherwise would be converted into cystine. A similar situation holds true with respect to the conversion of phenylalanine into tyrosine.

The amino acids in Group B, arginine and glycine, apparently can be synthesized by young mammals but often at a rate insufficient to permit maximum growth. On the other hand, both of these amino acids are necessary for normal growth of chicks. This is an instance where an amino acid may be dispensable for one species of animal and indispensable for another.

Knowledge concerning the amino acid requirements of man is somewhat limited.[58] Of the amino acids listed above as indispensable for the young animal, histidine is apparently dispensable from the diet of the adult human,[59] at least as judged by the criterion of maintenance of nitrogen equilibrium (see p. 1020). It is quite probable that as more specific criteria than growth or nitrogen balance are used, a somewhat different picture will be obtained than that described here. The conservative policy in human nutrition would indicate the presence in the diet of adequate amounts of all the amino acids listed in the first three columns above, even though experiments covering a short period in the life span may indicate that all of them are not needed for the period tested.

Three suggestions concerning the recommended daily intake of indispensable amino acids for human nutrition have been made. One is calculated from Rose's data on twice the minimum daily intake of each amino acid required to induce nitrogen equilibrium in young healthy adult men, another by Block and Bolling, calculated from the estimated per capita consumption of the principal protein foods in the United States in 1944, and the third from experiments on young soldiers on the low-calorie (900 calories per day) army survival ration.[60] These suggestions are summarized below.

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[58] For review see Melnick: *J. Am. Dietet. Assoc.*, **19**, 685 (1943).

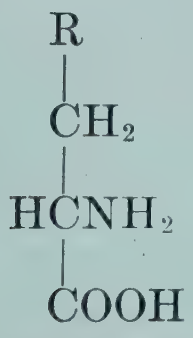
[59] Rose, Haines, and Warner: *J. Biol. Chem.*, **206**, 421 (1954).

RECOMMENDED DAILY CONSUMPTION OF INDISPENSABLE AMINO ACIDS (AVERAGE FOR ENTIRE POPULATION)

Amino Acid	*From Rose*[61] *(g. per day)*	*Block and Bolling (g. per day)*	*U.S. Army Survival Ration (g. per day)*
Arginine		4.8	3.1
Histidine		2.1	0.7
Lysine	1.6	4.7	3.3
Tryptophan	0.5	1.1	1.0
Phenylalanine	2.2	5.0	5.5*
Methionine	2.2	3.6†	3.8†
Threonine	1.0	3.4	2.6
Leucine	2.2	8.3	4.2
Isoleucine	1.4	5.3	3.5
Valine	1.6	5.3	4.3

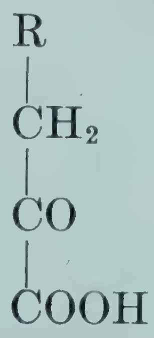
* Includes tyrosine.
† Includes cystine.

Origin of Amino Acids. Although plants are able to synthesize all the known amino acids when supplied with a source of nitrogen, inorganic as well as organic, the higher animals at least are able to synthesize only a little more than one-half of the common amino acids from the ordinary constituents of the diet or tissues. The limiting factor in the case of those amino acids which cannot ordinarily be synthesized appears in many instances to be the carbon chain or ring structure and not the nitrogen, since it is frequently found experimentally that the synthetic keto- or hydroxy-acid analog of the amino acid will take the place of the latter in the diet:



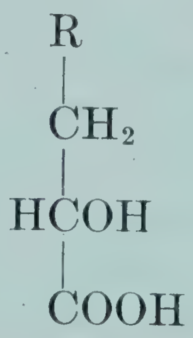
$$\begin{array}{c} R \\ | \\ CH_2 \\ | \\ HCNH_2 \\ | \\ COOH \end{array}$$

Amino acid



$$\begin{array}{c} R \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{array}$$

Keto acid



$$\begin{array}{c} R \\ | \\ CH_2 \\ | \\ HCOH \\ | \\ COOH \end{array}$$

Hydroxy acid

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[60] Spector: *Nutrition Revs.*, **10**, 289 (1952).

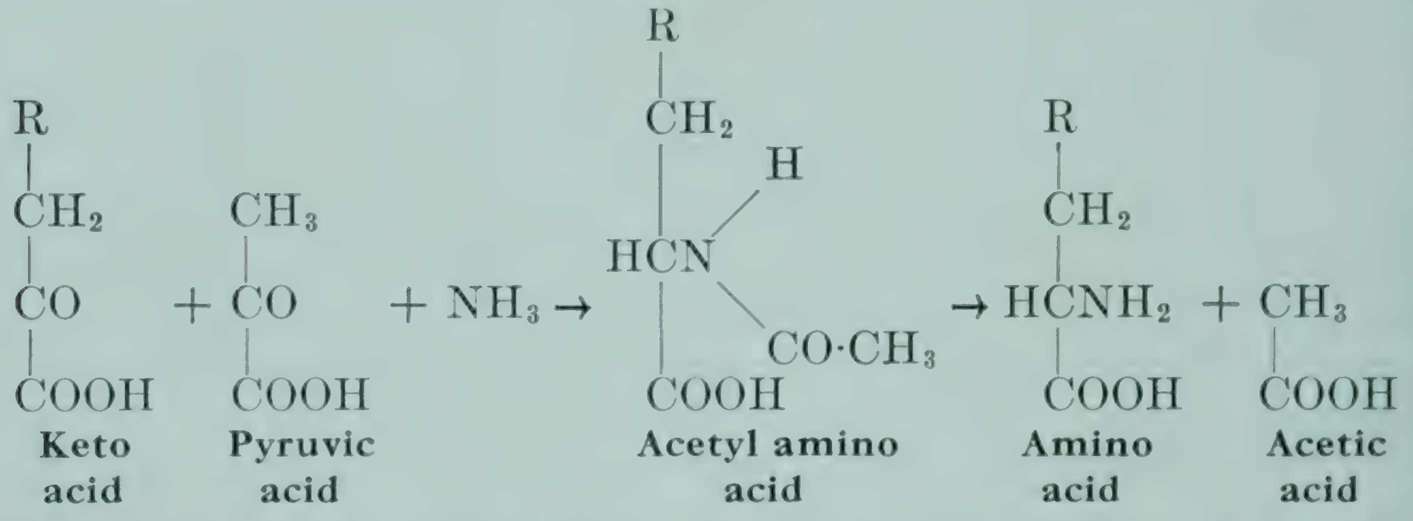
[61] It is not believed that these values, which are only twice the minimum daily requirements of young healthy adult males, should be used as the recommended amino acid intakes of the entire American population.

Sometimes, but not always, both keto acid and hydroxy acid are effective in this respect; the hydroxy acid presumably gives rise to the keto acid by oxidation. Replacement of a dietary amino acid by its corresponding keto acid indicates that metabolism of the amino acid proceeds reversibly through the keto acid stage.

Synthesis of an amino acid from a keto acid usually is considered to involve a reversible equilibrium with ammonia; this may proceed through the intermediate formation of the imino acid:

$$\underset{\textbf{Amino acid}}{R\cdot CH_2\cdot CH(NH_2)\cdot COOH} \underset{-2H}{\overset{+2H}{\rightleftarrows}} \underset{\textbf{Imino acid}}{R\cdot CH_2\cdot C{:}NH\cdot COOH} \underset{+H_2O}{\overset{-H_2O}{\rightleftarrows}} \underset{\textbf{Keto acid}}{R\cdot CH_2\cdot CO\cdot COOH + NH_3}$$

The biological formation of the amino acid from the keto acid may also involve acetylation (du Vigneaud and Irish):[62]



Another mechanism for the formation of amino acid from keto acid is by the transamination reactions described on p. 1024. It is not possible at the present time to evaluate the relative significance of these various processes in the synthesis of amino acids within tissues.

The amino acids found in animal tissues are all of the L configuration (see Chapter 4). In the case of certain amino acids it has been found that the "unnatural" or D configuration is convertible into the natural form by the body. This presumably involves loss of the asymmetry around the α carbon by conversion of the amino acid to the keto acid, followed by asymmetric synthesis of the amino acid in its natural configuration. Enzymes capable of converting D amino acids to keto acids are found in many animal tissues. In supplying the amino acid needs of an animal or human with the synthetic DL amino acid, however, it is usually assumed that the D component will be unavailable and twice as much of the DL mixture is supplied as compared to the requirement for the L form. For some amino acids the D form is largely excreted in the urine unchanged, but there are instances (e.g., serine) where the D amino acid has been shown to produce toxic manifestations.

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[62] du Vigneaud and Irish: *J. Biol. Chem.*, **122**, 349 (1937). See also Bloch and Borek: *J. Biol. Chem.*, **164**, 483 (1946).

Daily Protein Requirement. The amount of protein required by the individual per day is a nutritional factor of obvious practical significance. A variety of conditions will clearly influence estimations of the protein requirement. Among these may be mentioned growth, pregnancy, or other special demands of the individual; digestibility and absorbability and amino acid composition of the protein; possibly economic availability, individual idiosyncrasy, etc. Estimates of the protein requirement of man usually have been based on studies of the nutritional status of groups of individuals on varying kinds and amounts of protein, and on the extension of the results of animal feeding experiments to man. The Food and Nutrition Board of the National Research Council has recommended that the protein intake of an adult be 1.0 g. per kg. per day, of good quality protein, for adequate nutrition. This corresponds to about 65 g. per day for an average adult human, and will result in a daily urinary nitrogen excretion of about 10 g. The recommended protein allowance for the female is increased during pregnancy and lactation; and for children it varies with age.[63]

The efficiency with which a given protein supplies the nitrogen requirements of an animal may be defined in terms of its so-called "biological value." Although the methods used in the past for the determination of biological value may be subject to revision in the light of changing concepts of protein metabolism (see p. 1048), the concept itself retains its usefulness. Broadly speaking, a protein of high biological value is one which has a high digestibility and absorbability and supplies the organism with adequate amounts of those amino acids which it needs. The amino acids will include not only those which cannot be synthesized by the animal, but also sufficient of the dispensable amino acids to minimize the requirements for their synthesis. The presence of low or inadequate amounts of even one of the indispensable amino acids may be the limiting factor in the biological value of a protein. For example, if one such amino acid were present in a protein in such low amount that at ordinary levels of ingestion of the protein only half the animal's requirement for this amino acid were met, a nutritional deficiency would result unless the protein intake were raised considerably above the usual level, thus leading to a lowered efficiency with respect to utilization of the other amino acids present. In general, animal proteins are found to have a higher biological value than plant proteins because of the more satisfactory distribution in kind and amount of the various amino acids present. Endosperm proteins of the cereal grains (corn, wheat, rice) are lower in certain of the indispensable amino acids, particularly lysine, than are the commonly consumed proteins of animal origin. This has led to the erroneous generalization that *all* plant proteins are incomplete or poorly balanced with respect to their essential amino acid composition; as a matter of fact, many vegetable proteins, such as those of oats, beans, yeasts, wheat and corn embryos, leafy vegetables, and grasses, are almost as suitable sources of the amino acids as many of the more expensive animal products. It is sometimes recommended that at least half the protein of the diet be of animal origin. This is not necessarily an adequate criterion *per se*, and several proteins of low biological value individually may so complement one another with regard to amino acid composition as to provide a mixture of high biological value provided that they are ingested simultaneously.

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[63] See table, p. 1108.

Since the absence of any one of the indispensable amino acids will result in incomplete retention (utilization) of the remainder, it has been proposed[64] that the biological value of certain proteins may be related not only to their content of indispensable amino acids but to the relative rates of their release in and absorption from the intestinal tract. For example, the biological value of soy protein is increased when the raw soybean meal is autoclaved, while that of casein is diminished by baking; in neither case is any difference in indispensable amino acid content observed by analysis. Nevertheless it has been demonstrated that the processing of soy protein increases the rates of release of methionine, leucine, and lysine during enzymatic digestion but at relatively different rates. The effect of differential rates of absorption *in vivo* would be to furnish incomplete mixtures of absorbed amino acids at the early stage of absorption followed by the too late release and absorption of the supplementary amino acids needed for most efficient utilization. It is well known that ingested amino acids do not accumulate in the blood stream. Hence the proteins of highest biological value are those that not only contain the essential amino acids in adequate amounts but make them available for absorption at relative rates consistent with most efficient utilization for protein synthesis and retention.

Nitrogen Balance and Nitrogen Equilibrium. The relation between the amount of nitrogen entering the body from the diet in the form of amino acids, and the amount of nitrogen excreted from the body in the form of metabolic end products (chiefly as urea, but to some extent as uric acid, creatinine, etc.) is known as the nitrogen balance. The nitrogen balance is positive if intake exceeds output, negative if output exceeds intake, and if intake and output are essentially equal, nitrogen equilibrium results. The young growing animal must be in positive nitrogen balance, since a certain portion of the ingested nitrogen is retained as newly formed body proteins and nonprotein nitrogenous compounds. During fasting, or illness associated with wastage of tissues, a negative nitrogen balance exists. The normal healthy adult is ordinarily in a state of nitrogen equilibrium. That is, if the dietary intake of nitrogen is, say, 15 g. per day, the total nitrogen excreted by all channels (urine, feces, skin) will be approximately 15 g. Of this excreted nitrogen, the urine ordinarily contributes about 90 per cent.

Nitrogen equilibrium may be established at almost any desired level of nitrogen intake, from as low as 2 g. per day to 25 or 30 g. or even more. This apparently is because within these limits the intensity of nitrogen metabolism is determined by the rate of entrance of nitrogen into the body. It was thought at one time that equality of nitrogen intake and excretion represented essentially a disposal of dietary nitrogen in excess of the needs of the animal; that is, a small portion of the entering nitrogen was considered to be utilized by the body for the replacement of nitrogen loss due to wear and tear on the body tissues, but the bulk of dietary nitrogen was essentially surplus material and was promptly utilized for energy-yielding purposes without contributing to the nitrogen metabolism of the tissues. This was the basis for the classical distinction of Folin between endogenous and exogenous nitrogen metabolism; according to this concept, the bulk of excreted nitrogen was of exogenous origin and did not arise by the metabolism of tissue protein.

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[64] Melnick, Oser, and Weiss: *Science*, **103**, 326 (1946).

It is now known that this concept is not true, and that in general the nitrogenous constituents of the diet promptly enter into the varied nitrogen and protein metabolism of the body tissues, becoming indistinguishable from similar substances already present, and the equality between nitrogen intake and output is due primarily to the fact that the rate at which a certain amino acid is metabolized is determined largely by the rate at which that amino acid becomes available to the tissues. In other words, the entrance of a given amount of amino acid into the metabolic processes of the tissue brings about the metabolism of an equal amount of the amino acid molecules which are already present. Thus the distinction between endogenous and exogenous breakdown disappears, and must be replaced by the concept of a continuous and dynamic nitrogen metabolism the rate of which is determined, as are all chemical reactions, by the concentration of reactants present at a particular time. The only biological exception to this thus far discovered is in connection with purine metabolism; apparently dietary purines cannot penetrate the cell nucleus and thus enter into the endogenous metabolism of nuclear purines.[65]

This overthrow of the classical distinction between endogenous and exogenous metabolism is due largely to the pioneer work of Schoenheimer and his associates, based upon the application of isotopes to biological problems. The details of some of this work are instructive. Various amino acids (e.g., leucine, glycine) were synthesized in the presence of isotopic nitrogen (N^{15}) so that the amino acid contained a significant amount of the isotope. These amino acids were then incorporated in small amount in the diet of rats. The urine was collected over a three-day period, after which the animals were sacrificed and the body nitrogen fractionated into protein and nonprotein portions. These, as well as the excreta, were then analyzed for the presence of the isotopic nitrogen. According to the classical concept of endogenous and exogenous metabolism, the urinary urea should have contained most of the dietary nitrogen and therefore most of the isotopic nitrogen should have appeared promptly in the urine. This did not happen. Less than one-half of the isotopic nitrogen of the glycine was excreted, and less than one-third of that of the leucine. The bulk of the unexcreted isotopic nitrogen was found in the tissue proteins. Later experiments have shown that the labeled amino acid is to a certain extent incorporated directly into the tissue proteins, and likewise contributes its nitrogen to various other amino acids of the body, since both the fed amino acid and other amino acids isolated from the tissue proteins were proved to contain the isotope.

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[65] Plentl and Schoenheimer: *J. Biol. Chem.*, **153**, 203 (1944).

In general, therefore, one may state that the amino acids of the diet enter rapidly into biological equilibrium with the amino acids of the body, becoming incorporated into newly formed protein or entering into reactions which supply nitrogen for the synthesis of other amino acids or other nitrogenous constituents of the tissues. These reactions occur more or less rapidly among the various tissues and somewhat independently of the nutritional state of the animal; labeled amino acids are found incorporated into the animal body proteins both when there is an abundance of that particular amino acid in the diet, and when on a nitrogen-free diet the tissue proteins are being extensively broken down for energy purposes.

Conversion of Protein to Carbohydrate and Fat. It has been well established that after metabolic removal of nitrogen, the carbon chain of certain of the amino acids may be utilized by the animal for the formation of carbohydrate. The classical basis for demonstrating this formation entails the use of the drug phlorizin. If an animal is treated with phlorizin, the renal threshold for glucose is lowered to such a degree that administered glucose, or that formed within the animal body by metabolic processes, is excreted almost quantitatively in the urine ("phlorizin diabetes"). A fasting phlorizinized animal will continue to excrete glucose in the urine long after all carbohydrate stores have been exhausted. It is usually (but not invariably) found that such an animal excretes about 3.6 g. of glucose for every gram of urinary nitrogen. The ratio of urinary glucose (dextrose) to urinary nitrogen is known as the D/N ratio, which in this instance is 3.6.

A D/N ratio of 3.6 usually is interpreted to mean that out of every 100 g. of body protein metabolized by the fasting animal, which would correspond to the excretion of 16 g. of urinary nitrogen, about 58 g. of glucose are formed, since $^{58}/_{16} = 3.6$. The remaining 26 g. of protein, i.e., $100 - (58 + 16)$, presumably represents that portion which is metabolized via fatty acid or ketone body formation. It should be noted here that there are some who disagree with this interpretation of the significance of the D/N ratio.

If to a fasting phlorizinized animal either protein or certain amino acids are administered, extra glucose is found in the urine, along with extra metabolic nitrogen. By quantitative measurement it is believed possible to evaluate the ability of either protein or amino acids to yield glucose in the animal body. Using this method, it has been found, for example, that various proteins yield from about 50 to as high as 80 per cent of their weight as glucose, and that certain amino acids are glucose-formers while others are not. Those amino acids which have been shown to be glucogenic by this procedure include glycine, alanine, cysteine, methionine, norleucine, proline, serine, valine, arginine, aspartic acid, and glutamic acid. Those amino acids which do not yield extra glucose in the phlorizinized animal include isoleucine, leucine, lysine, phenylalanine, tyrosine, and tryptophan.

The ability of protein to yield glucose would thus appear to be related to the relative proportion of glucogenic amino acids in the molecule and wide variation among proteins may be expected. It is interesting to note that there is a rough parallelism between the glucogenic amino acids and those capable of being synthesized within the animal body. In the light of current knowledge concerning intermediary carbohydrate and protein metabolism, it is not surprising that such amino acids as alanine, glutamic acid, and aspartic acid are glucogenic, since after metabolic removal of their nitrogen there remain the substances pyruvic acid, ketoglutaric acid, and oxalacetic acid respectively, and these latter compounds are recognized intermediates in carbohydrate breakdown and synthesis.

Other methods of studying the conversion of amino acids to carbohydrate include the use of perfusion through isolated organs; the ability of the compound to lead to increased liver glycogen content when administered to the fasting rat; and the ability of the amino acid to reduce an experimentally induced ketosis. Results by the various methods described are not always concordant for a particular amino acid. Tryptophan, for example, will reduce an experimental ketosis but will not lead to an increased liver glycogen content or an increased excretion of glucose in the phlorizinized dog; and other examples might be cited. It is possible that these various procedures measure metabolically independent functions rather than the same general property of carbohydrate formation; but more must be learned about the individual reactions concerned before the subject will be better understood.

The conversion of amino acids to fats or fat metabolites is less well understood than that of carbohydrate formation. Certain of the amino acids (e.g., tyrosine, phenylalanine, leucine) appear to be oxidized directly by way of the formation of acetoacetic acid, and this may in turn give rise to the synthesis of fatty acids. Since proteins can be converted into glucose, and glucose into fat, some fat may arise from protein in this fashion. The significance of this in normal nutrition is uncertain.

Protein Storage. There is as yet no evidence for the existence within the animal body of a storage form of protein analogous to the storage of carbohydrate as glycogen, or of fat. Yet there does appear to be a reserve protein supply which can be drawn upon to furnish the fundamental nitrogen requirements of the animal when the protein intake is inadequate. This reserve protein appears to be drawn from the tissues themselves; such organs as the liver and kidney, and the blood plasma, appear to be capable of undergoing a considerable depletion of protein content to supply the needs of other parts of the body for nitrogen during fasting. Other organs—e.g., brain—are more resistant to protein depletion.

Specific Dynamic Action of Protein. When protein is fed, more heat is produced in the body than can be accounted for by the combustion of the protein ingested. One view is that this action is due to the stimulating action on the tissue cells of certain products of amino acid catabolism, perhaps hydroxy acids, leading to increased oxidation of carbohydrate by such cells. Glycine, alanine, and phenylalanine appear responsible for much of the specific dynamic action of proteins. Carbohydrates and fats also exert a specific dynamic action, but to a lesser extent than proteins and amino acids. For a further discussion of this subject, the reader is referred to Borsook[66] and Kriss.[67]

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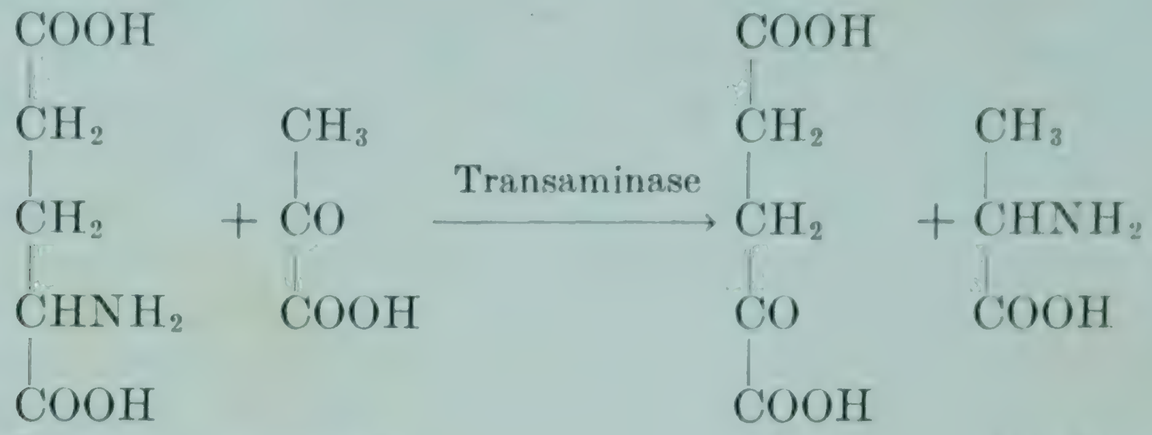
[66] Borsook: *Biol. Revs.*, **11**, 147 (1936).

[67] Kriss: *J. Nutrition*, **21**, 257 (1941).

General Physiological Transformation of Amino Acids. The amino acids in the body undergo many varied and complicated reactions. Some of these are being revealed in detail as a result of the extensive use of isotope techniques.

TRANSAMINATION. This refers to the transfer of amino groups from one compound to another. An amino acid under the influence of a specific enzyme loses its amino group to a keto acid and the original compound becomes a keto acid.

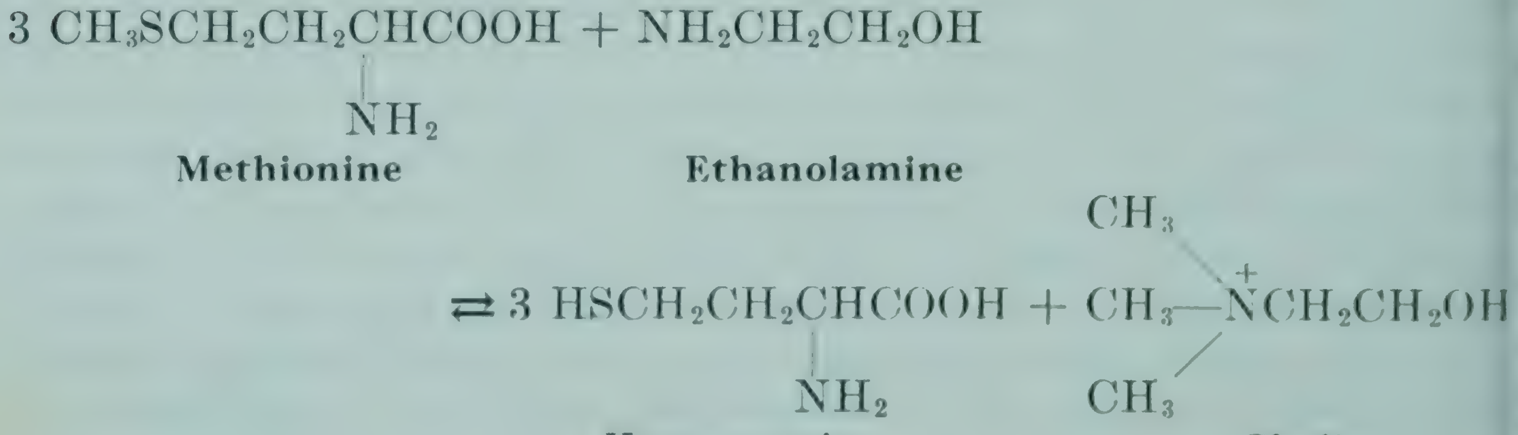


$$\begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array} + \begin{array}{c} CH_3 \\ | \\ CO \\ | \\ COOH \end{array} \xrightarrow{\text{Transaminase}} \begin{array}{c} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CO \\ | \\ COOH \end{array} + \begin{array}{c} CH_3 \\ | \\ CHNH_2 \\ | \\ COOH \end{array}$$

Glutamic acid **Pyruvic acid** **α-Keto-glutaric acid** **Alanine**

The keto acids found in these reversible reactions are concerned in oxidation-reduction systems (carbohydrate metabolism) and thus may furnish amino acids or carbohydrate intermediates as required, i.e., transamination is one link between protein and carbohydrate intermediary metabolism.

TRANSMETHYLATION. When methyl groups are linked to the nitrogen or sulfur of certain organic compounds they are capable of being shifted intact among these substances (du Vigneaud).



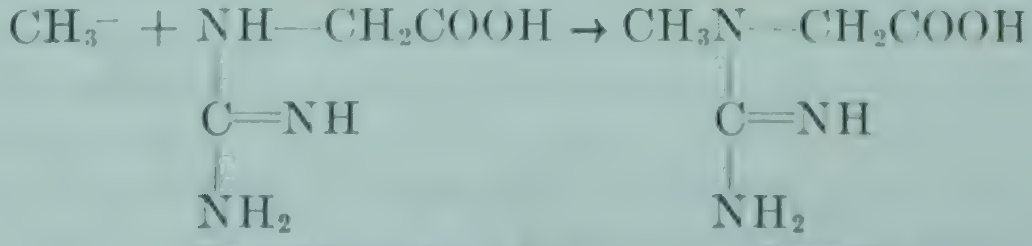
$$3\ CH_3SCH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH + NH_2CH_2CH_2OH$$

Methionine **Ethanolamine**

$$\rightleftarrows 3\ HSCH_2CH_2\underset{\displaystyle NH_2}{\underset{|}{C}}HCOOH + CH_3-\overset{CH_3}{\underset{CH_3}{\overset{+}{N}}}CH_2CH_2OH$$

Homocysteine **Choline**

These so-called labile methyl groups[68] may then react with other labile methyl group substances (guanidoacetic acid, nicotinic acid, purines, etc.) to methylate these compounds.



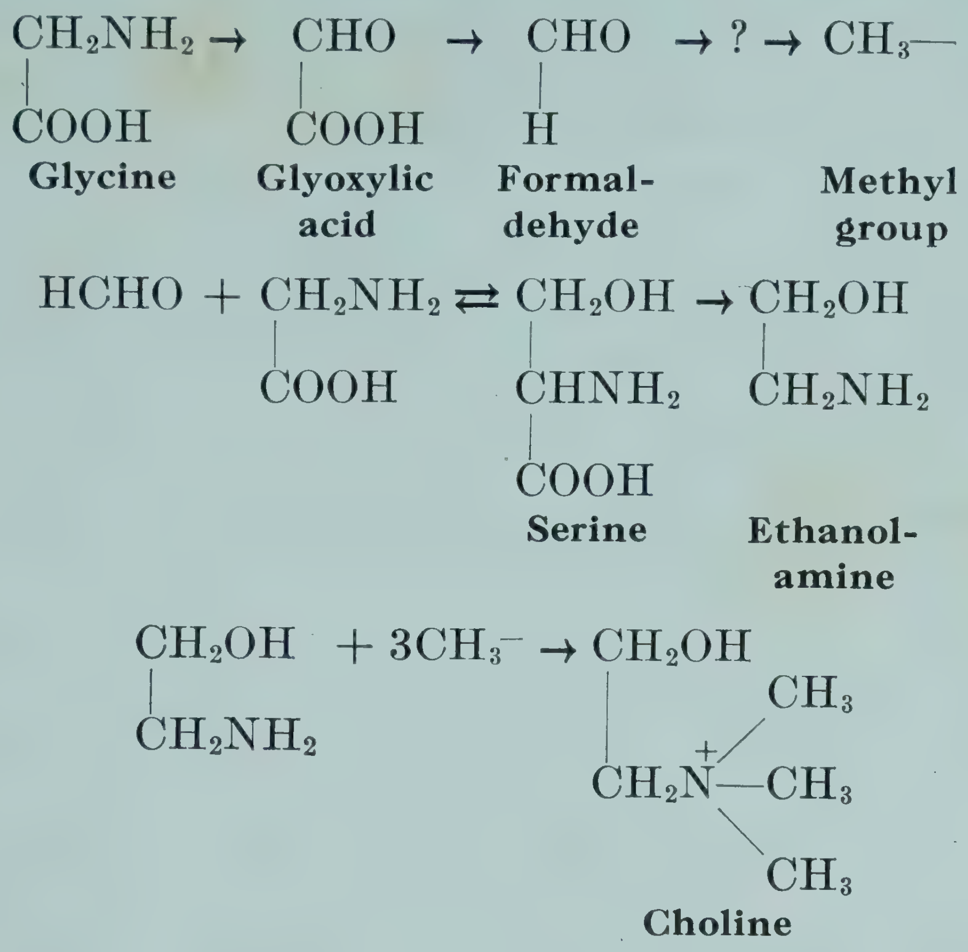
$$CH_3^- + \begin{array}{l} NH-CH_2COOH \\ | \\ C{=}NH \\ | \\ NH_2 \end{array} \rightarrow \begin{array}{l} CH_3N-CH_2COOH \\ \quad | \\ \quad C{=}NH \\ \quad | \\ \quad NH_2 \end{array}$$

Guanidoacetic acid **Creatine**

---

[68] See p. 1029.

However, the methyl groups now become fixed and are no longer able to transmethylate. Further aspects of transamination and transmethylation are given on p. 1034 and p. 1029. Reactions thought to be concerned are given here:

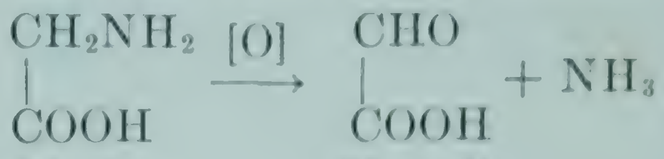


METABOLISM OF INDIVIDUAL AMINO ACIDS

Glycine. This amino acid enters into a variety of metabolic functions. In addition to its presence in many of the body proteins (collagen, for example, is rich in glycine), it is concerned in the synthesis of such non-protein compounds as (1) the glycocholic acid of the liver, (2) the creatine of muscle and other tissues, (3) the glutathione of the cells, (4) the protoporphyrin (heme) portion of hemoglobin, (5) the hippuric acid and similar compounds found in the urine after ingestion of benzoic acid and other unoxidizable substances, and (6) the methyl group of choline, methionine, etc. Other aspects of glycine metabolism undoubtedly remain to be recognized.

Glycine is readily synthesizable by both the rat and the adult human; it is thus "dispensable" from the diet of these species. It has been shown, however, that about 1 per cent of glycine is necessary in the diet of the chick to ensure adequate nutrition (Almquist). This is one of several known examples of species specificity with regard to dietary amino acid requirements. The rate of synthesis of glycine in man has been estimated by Quick, on the basis of the rate of excretion of hippuric acid after benzoate administration, to be somewhat over 0.5 g. per hour. It has been shown by Rittenberg and Schoenheimer that synthesized glycine may be derived from sources within the body as well as from the diet. It has been demonstrated that the amino acid serine is a significant precursor of glycine, and there is some indication that glutamic acid may also give rise to glycine, possibly through the intermediary formation of serine.

Knowledge concerning the metabolic degradation of glycine is extensive; only a small portion will be reviewed here. In the diabetic dog, the carbon chain of glycine can be shown to be readily convertible into glucose. Animal tissues contain a flavoprotein enzyme which catalyzes the oxidation of glycine to glyoxylic acid and ammonia.

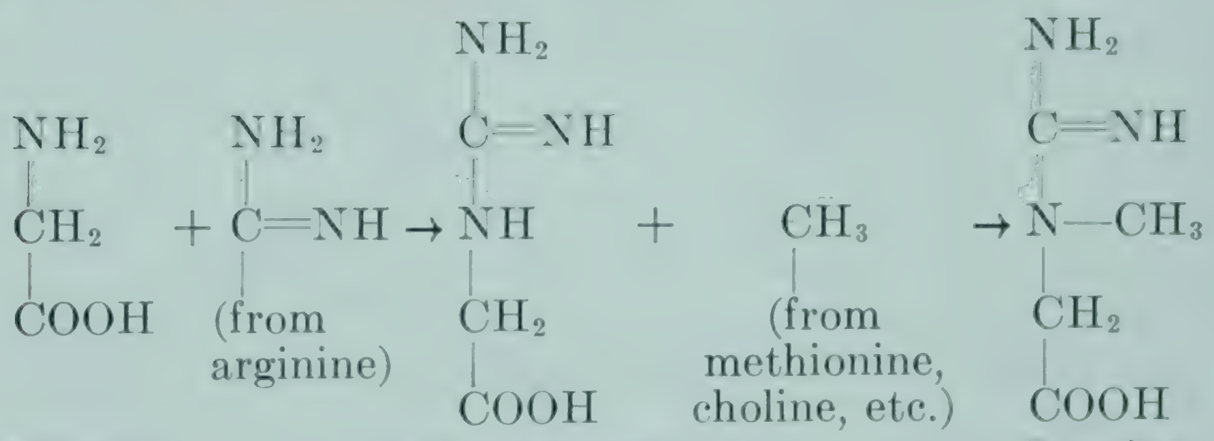


$$\begin{array}{l}CH_2NH_2\\ |\\ COOH\end{array} \xrightarrow{[O]} \begin{array}{l}CHO\\ |\\ COOH\end{array} + NH_3$$

Glycine **Glyoxylic acid**

This reaction may be of importance in connection with the possible formation of labile methyl groups by way of formaldehyde, as postulated on p. 1025.

Of major interest is the part played by glycine, along with arginine and a labile methyl group, in the biological synthesis of creatine. The separate steps in the formation of creatine have been shown to be as follows:



$$\begin{array}{l}NH_2\\ |\\ CH_2\\ |\\ COOH\end{array} + \begin{array}{l}NH_2\\ |\\ C{=}NH\\ |\\ \text{(from arginine)}\end{array} \rightarrow \begin{array}{l}NH_2\\ |\\ C{=}NH\\ |\\ NH\\ |\\ CH_2\\ |\\ COOH\end{array} + \begin{array}{c}CH_3\\ |\\ \text{(from methionine,}\\ \text{choline, etc.)}\end{array} \rightarrow \begin{array}{l}NH_2\\ |\\ C{=}NH\\ |\\ N{-}CH_3\\ |\\ CH_2\\ |\\ COOH\end{array}$$

Glycine **Guanidoacetic acid** **Creatine**

Bloch and Schoenheimer[69] demonstrated by feeding experiments using both glycine and arginine containing isotopic nitrogen that the two nitrogen atoms in the amidine portion ($-\overset{NH_2}{C}=NH$) of creatine were derived from the amidine portion of arginine, and the third nitrogen atom came from glycine. Borsook and Dubnoff[70] showed that kidney tissue appears to form guanidoacetic acid from arginine and glycine, and that liver tissue probably synthesizes creatine from guanidoacetic acid in the presence of methionine. Guanidoacetic acid itself, the intermediate in creatine synthesis, is found normally in only small amounts in animal tissues, but readily gives rise to extra creatine formation when fed. Glycine appears also to be directly concerned in the synthesis of glutathione, the tripeptide which is thought to be of importance in intracellular oxidation and reduction. It has been shown that when isotopic glycine is fed to an animal, the labeled glycine appears so rapidly in the glutathione of the animal body as to indicate the direct participation of glycine in the synthesis of this compound. Involvement of glycine in the synthesis of hemin by the adult human is also indicated by the use of isotopic glycine.

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[69] Bloch and Schoenheimer: *J. Biol. Chem.*, **138**, 167 (1941).

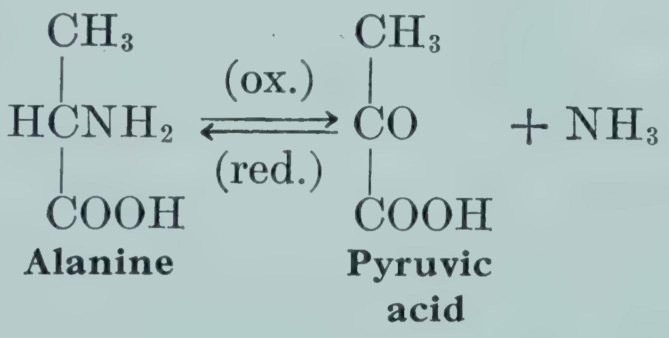
[70] Borsook and Dubnoff: *J. Biol. Chem.*, **132**, 559 (1940); **138**, 389 (1941).

After prolonged administration of labeled glycine to an individual, the hemin isolated from the red blood cells contains sufficient isotopic carbon to indicate a major role of glycine in the synthesis of the pyrrole ring of the hemoglobin molecule. In fact the formation of heme from glycine has been found by *in vitro* studies in which glycine labeled with N^{15} and C^{14} was incubated with red blood cells of sickle-cell anemic patients or with the nucleated red cells of birds. Acetic acid also has been implicated in this synthesis (see p. 1009).

Although it was formerly believed that the animal was unable to synthesize methyl groups and thus was forced to obtain preformed methyl groups for creatine, etc. from methionine or choline, it has now been demonstrated that many compounds are capable of supplying this metabolic essential. Thus choline, betaine, and methionine are able to contribute labile methyl groups[71] to each other and to guanidoacetic acid by the process of transmethylation, while glycine, serine, methylol, formic acid, formaldehyde, and others are sources of methyl groups by conversion to an unknown one-carbon intermediate.

Relatively little work has been done on the role of glycine in the synthesis of the glycocholic acid of the liver. The relationship of glycine to hippuric acid formation is discussed in detail in Chapter 20. The ability of certain tissues to synthesize hippuric acid *in vitro* has been used as a means of establishing possible precursors of glycine.

Alanine. This 3-carbon amino acid is readily synthesized by the animal body and hence is dispensable from the diet. Metabolism and synthesis are usually considered to involve the reversible equilibrium of oxidative deamination (see p. 1018) and transamination (see p. 1034). The reaction of oxidative deamination is as follows:



$$\begin{array}{c} CH_3 \\ | \\ HCNH_2 \\ | \\ COOH \\ \textbf{Alanine} \end{array} \underset{\text{(red.)}}{\overset{\text{(ox.)}}{\rightleftarrows}} \begin{array}{c} CH_3 \\ | \\ CO \\ | \\ COOH \\ \textbf{Pyruvic acid} \end{array} + NH_3$$

There is little evidence that this reaction actually occurs in the tissues; the L-amino acid oxidase of Blanchard, *et al.*,[72] attacks alanine but slowly, and there is no biological evidence for the synthesis of alanine from pyruvic acid and free ammonia. A more plausible metabolic pathway is by the reactions of transamination described on p. 1034, whereby synthesis would entail the transfer of the amino group of either glutamic or aspartic acid to pyruvic acid, to yield alanine and either ketoglutaric acid or oxalacetic acid, and metabolic breakdown would be the reverse of these reactions. The pyruvic acid thus formed from alanine by transamination may then follow the normal course of oxidation of carbohydrate intermediates (p. 990). Conversion to pyruvic acid presumably is the explanation for the glucogenic action of alanine. By the use of alanine labeled with isotopic hydrogen (deuterium), it has been shown that alanine may be a source of acetyl groups in the acetylation of certain foreign amino acids excreted in the urine as acetyl derivatives (see p. 1012 and Chapter 20).

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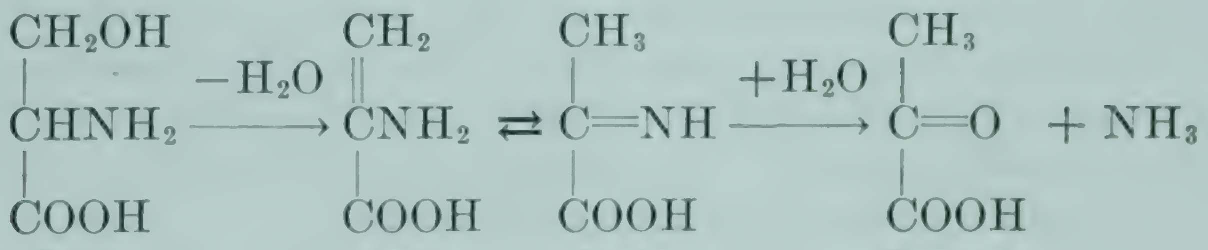
[71] See p. 1029.

[72] Blanchard, Green, Nocito, and Ratner: *J. Biol. Chem.*, **161**, 583 (1945).

Serine. This hydroxy-amino acid is readily synthesizable by the animal body and is thus dispensable from the diet. It nevertheless participates in a somewhat unusual variety of metabolic reactions in the tissues. In addition to its presence in proteins, it has been found in phospholipide material, where it apparently serves in a manner analogous to choline and ethanolamine in the structure of phospholipide molecules (see Chapter 11). In fact, studies using serine labeled with isotopic nitrogen indicate that ethanolamine arises by the decarboxylation of serine; ethanolamine can then give rise to choline by methylation. Serine is also a precursor of glycine (see p. 1025).

Serine is also concerned in the formation of cystine (see p. 1031). Serine is glucogenic in the diabetic dog. The "unnatural" optical isomer of serine, D-serine, is toxic to rats. The nitrogen of serine has been shown by isotopic experiments to be incorporated into the N_7 of uric acid while the β-carbon appeared at C_2 and C_8 having been initially converted to formate.

The anaerobic deamination of serine appears to take the following course:



$$\begin{array}{ccccccccc} CH_2OH & & CH_2 & & CH_3 & & CH_3 & & \\ | & & \| & & | & & | & & \\ CHNH_2 & \xrightarrow{-H_2O} & CNH_2 & \rightleftarrows & C{=}NH & \xrightarrow{+H_2O} & C{=}O & + & NH_3 \\ | & & | & & | & & | & & \\ COOH & & COOH & & COOH & & COOH & & \end{array}$$

Serine **Amino acrylic acid** **Imino pyruvic acid** **Pyruvic acid**

Threonine. This amino acid cannot be synthesized by the animal body, and its presence in the diet in adequate amount is required both for growth in the young rat and for the maintenance of nitrogen equilibrium in the adult human. Threonine was the last "essential" amino acid to be discovered, in the sense that its isolation from casein and characterization by McCoy, Meyer, and Rose in 1935 first permitted the use of a mixture of pure amino acids as the sole source of nitrogen in an experimental diet.

Relatively little is known concerning the metabolism of threonine. It is glucogenic and antiketogenic. It is not attacked by the L-amino acid oxidase of rat kidney, but there may be another enzyme concerned since it has been reported to be deaminated by kidney tissue under anaerobic conditions to produce α-ketobutyric acid, $CH_3{\cdot}CH_2{\cdot}CO{\cdot}COOH$. Only the optically active form found naturally is utilizable for growth. Threonine and lysine are the only amino acids known which do not transaminate.

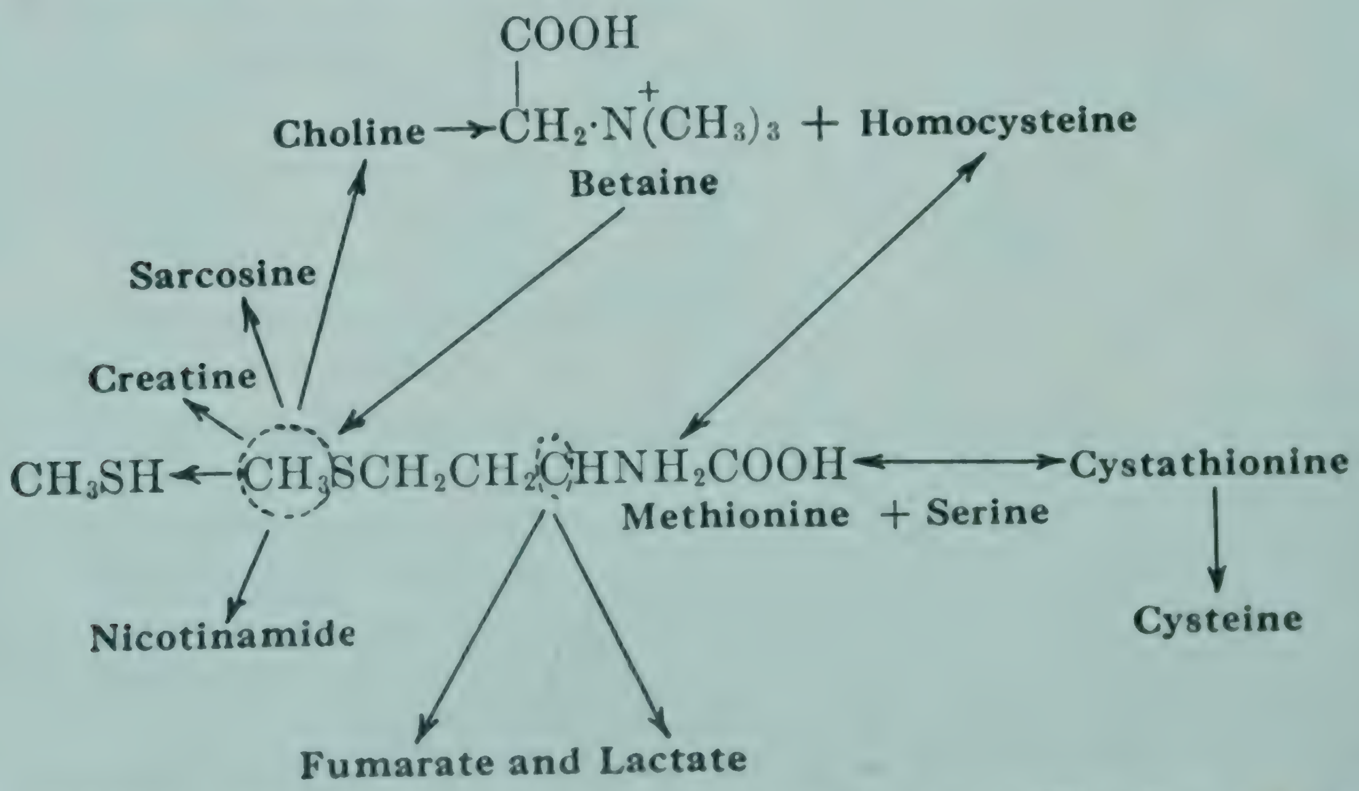
Methionine. This sulfur-containing amino acid cannot be synthesized by the animal body from the ordinary constituents of the diet, and must be present in adequate amount for the promotion of growth in the young animal and for the maintenance of nitrogen equilibrium in the adult human. Methionine alone will satisfy all of the sulfur requirements of the animal, since it is readily converted into cystine in the tissues, as described on p. 1031.

Methionine is glucogenic in the diabetic dog. Metabolism may proceed in part by deamination through the corresponding keto acid, $CH_3{\cdot}S{\cdot}CH_2{\cdot}CH_2{\cdot}CO{\cdot}COOH$, since this latter compound is formed to considerable extent on incubating methionine with liver tissue, and the young rat will grow on a diet containing the keto acid in place of methionine. Further stages in the oxidative breakdown of methionine are obscure. On complete oxidation, the sulfur is found in the urine as sulfate, either inorganic or ester, as with cystine (p. 1032).

TRANSMETHYLATION. An important metabolic function of methionine is in connection with the process known as transmethylation. It has been shown by du Vigneaud and associates that the animal body is able to use the methyl group of methionine to methylate certain nitrogen- and sulfur-containing compounds of the body. Methionine, choline, and betaine (trimethylglycine) have been shown to be suitable dietary sources of such methyl groups; of these, methionine appears to be quantitatively the most important. In the biological synthesis of creatine, for example (see p. 1026) the methyl group of creatine is derived by transfer from methionine. The process of methyl transfer from one compound to another is called *transmethylation*. A methyl group which may be so transferred is called a *labile methyl group;* thus far, only methyl groups attached to either nitrogen or sulfur appear to be labile and to participate in transmethylation. Contrary to the former opinion, labile methyl groups need *not* be supplied in the diet but can be synthesized from glycine, serine, formic acid, and their precursors (see p. 1025).

The role of methionine in transmethylation has been adequately proved by the use of methionine synthesized to contain isotopic hydrogen (deuterium) in the methyl group. When such labeled methionine is included in the diet of an animal, the methylated compounds choline and creatine subsequently isolated from the animal tissues prove to contain sufficient isotope to justify the conclusion that methyl groups have been transferred from the methionine to the other compounds in question. The ability of methionine to furnish methyl groups for the synthesis of choline from suitable precursors explains the lipotropic effect of this amino acid (see p. 1012). The transfer of methyl groups from methionine to choline is reversible, most probably through the intermediate formation of the amino acid homocysteine (see below); methylation of guanidoacetic acid to form creatine is irreversible, and the constant excretion of body creatine as urinary creatinine represents a loss of methyl groups from the body. The daily requirement for methyl groups, however, appears to be considerably in excess of such urinary loss, so that other pathways of methyl group degradation must be present; relatively little is known about this at the present time. Most of the evidence for transmethylation has been obtained with the rat, but the process has also been shown to occur in the adult human.

After removal of the methyl group from methionine, the amino acid homocysteine results. Homocysteine, $HS{\cdot}CH_2{\cdot}CH_2{\cdot}CH(NH_2){\cdot}COOH$, and its disulfide form homocystine (analogous to the relation between cysteine and cystine) are synthetic amino acids which have not as yet been isolated from natural sources. Their biological availability is such, however, as to lead to the belief that they represent normal intermediates in methionine metabolism. If an animal is placed on a diet containing no methionine but adequate amounts of homocysteine or homocystine, together with a source of labile methyl groups such as choline, betaine, or serine, the animal will synthesize the methionine it needs for normal growth. If the methyl groups of the dietary choline are labeled with deuterium, the methionine subsequently isolated from the tissue proteins proves to contain the isotope in its methyl group. The animal body is therefore capable of transferring methyl groups from choline via betaine to homocysteine, to form methionine; as has already been pointed out, this transfer is reversible. Since the reversible exchange of methyl groups between methionine and choline can be demonstrated not only on the homocysteine diet but also when the diet contains adequate amounts of methionine and choline, it is believed to represent a normal metabolic process of the tissues. The implication of homocysteine in the formation of cystine from methionine is discussed on p. 1031. The general metabolic relation between methionine, homocysteine, and related compounds may be represented as follows:

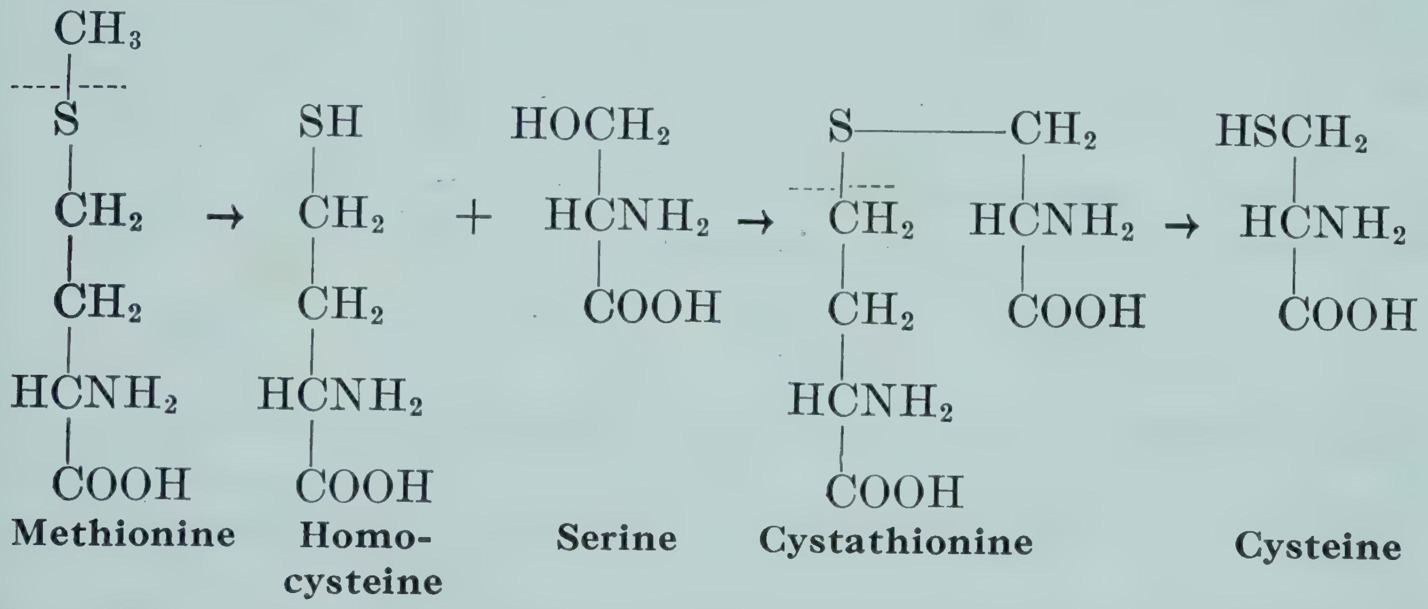


In addition to its known metabolic functions, methionine appears to play a specific part in protecting the liver from damage by such poisons as carbon tetrachloride, phosphorus, arsenic, and chloroform. The mode of action here is unknown.

Cystine and Cysteine. These two sulfur-containing amino acids are usually considered to be metabolically equivalent since one may be so readily converted into the other by oxidation or reduction. There is some evidence, however, that there may be biological differences between cysteine and cystine, particularly in specific organs such as the liver and kidney. In the rare metabolic abnormality known as *cystinuria*, individuals regularly excrete significant amounts of cystine in the urine. This excretion continues during fasting, and is not increased by the oral administration of cystine itself; the feeding of cysteine or of methionine does, however, augment cystine excretion. Thus the cystinuric distinguishes between orally administered cystine and cysteine, and the normal individual may do so in specific tissues as well.

It was thought for a long time that cystine was an indispensable amino acid, but Jackson and Block showed in 1932 that methionine could replace cystine for growth purposes in the rat on a low-cystine diet, and the ability of the animal body to form cystine from methionine is now well established. In 1939 Tarver and Schmidt demonstrated, by the use of methionine containing radioactive sulfur, that the sulfur of cystine was derived from the sulfur of methionine.

The mechanism of formation of cystine from methionine has been the subject of considerable study, and appears to have been finally established by the work of du Vigneaud and his associates as follows: methionine is demethylated to form the amino acid homocysteine; this condenses with serine to form an unsymmetrical thio-ether, called cystathionine; the cystathionine undergoes enzymatic cleavage so that the sulfur remains with the serine moiety, to produce cysteine. The fate of the remaining portion of the cystathionine molecule is as yet not known. These various steps may be illustrated as follows:



Evidence for the conversion of methionine to homocysteine has already been presented (p. 1029). When homocysteine and serine are incubated with liver tissue, cysteine is formed. It has also been shown by Stetten that when serine labeled with isotopic nitrogen is fed to animals, the cystine isolated from the tissues contains such a high proportion of the isotope as to indicate (but not prove) the direct conversion of serine to cysteine. Cystathionine, the postulated intermediate, has been found in the livers of fasting rats which were fed *both* L-methionine and L-serine.[73] If either amino acid were fed alone, no cystathionine was observed. If methionine is synthesized to contain both isotopic sulfur and isotopic carbon, and this doubly labeled compound is fed to rats, the cystine isolated from the hair proves to contain as much as 80 per cent of the isotopic sulfur, but no isotopic carbon.[74] This is further evidence that it is the sulfur and not the carbon chain of methionine which is involved in the formation of cystine.

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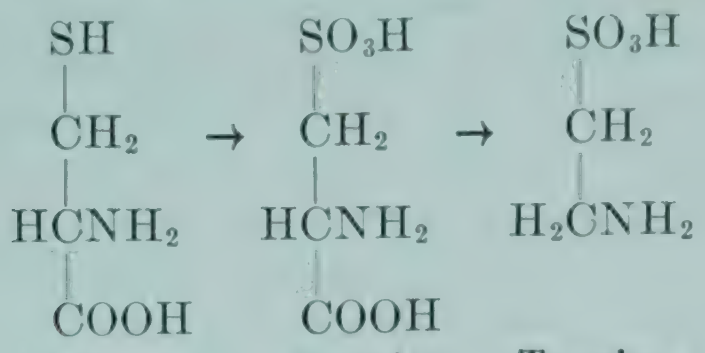
[73] Hess: *Arch. Biochem. Biophys.*, **40**, 127 (1952).

[74] du Vigneaud, Kilmer, Rachele, and Cohn: *J. Biol. Chem.*, **155**, 645 (1944).

Cystine and cysteine contribute to the formation of many important sulfur-containing compounds in the animal body. Cysteine is a component of glutathione (γ-glutamyl-cysteyl-glycine); and appears to be the precursor of the taurine of the taurocholic acid of the bile, probably through intermediate oxidation to form cysteic acid:

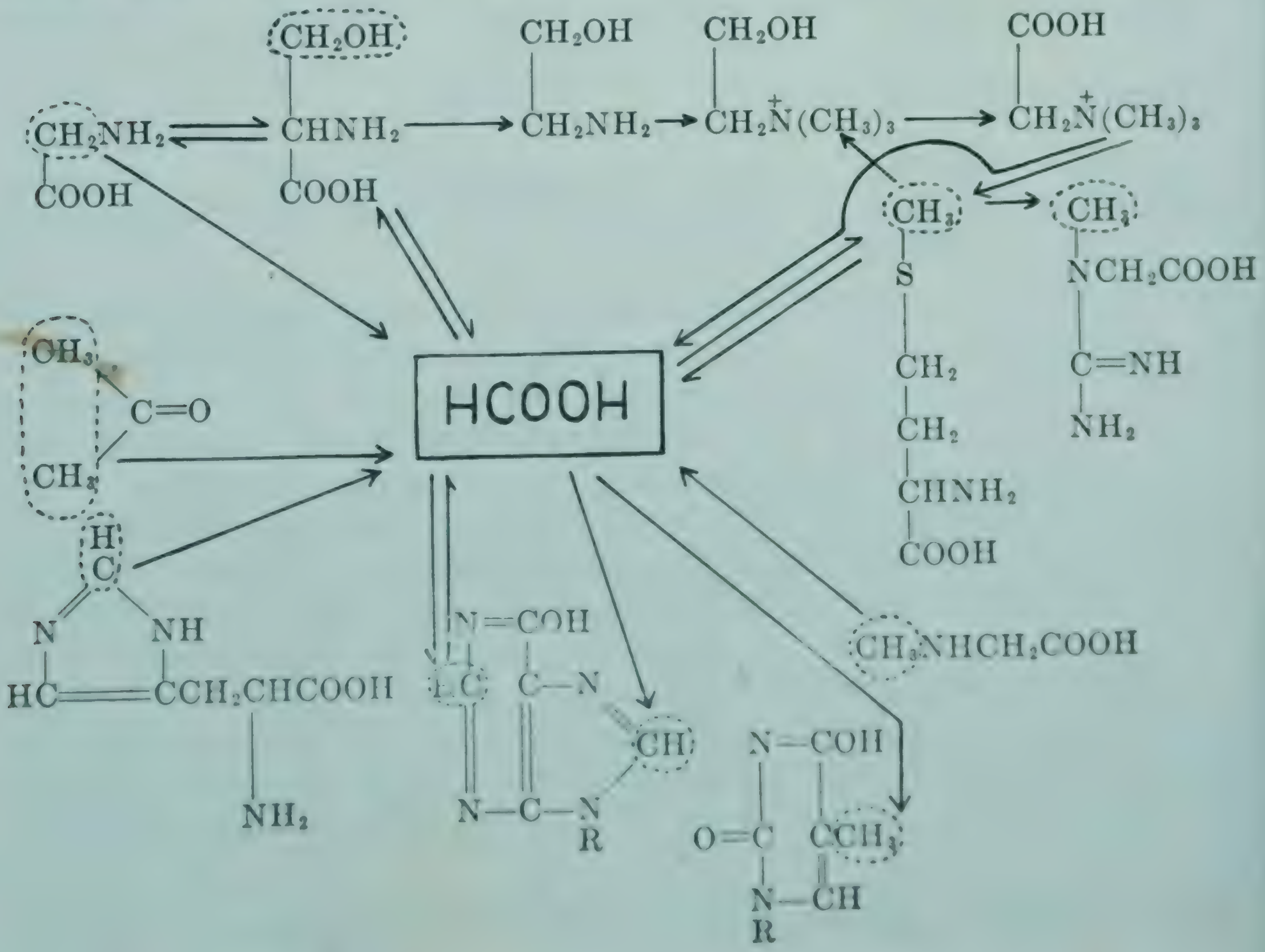


$$\begin{array}{ccccc} SH & & SO_3H & & SO_3H \\ | & & | & & | \\ CH_2 & \rightarrow & CH_2 & \rightarrow & CH_2 \\ | & & | & & | \\ HCNH_2 & & HCNH_2 & & H_2CNH_2 \\ | & & | & & \\ COOH & & COOH & & \end{array}$$

Cysteine **Cysteic acid** **Taurine**

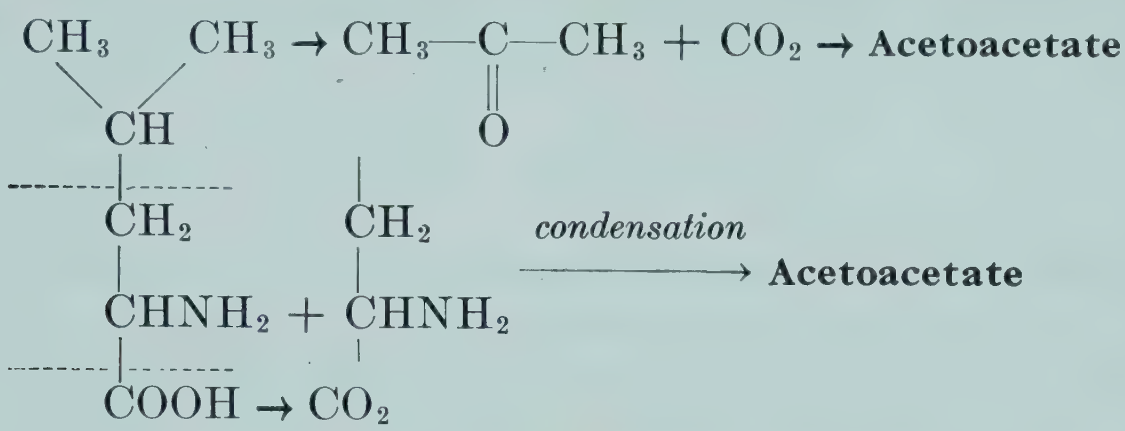
Cysteine is likewise found in the urine in combination with certain unoxidizable substances (detoxication), to form what are called mercapturic acids (see Chapter 20). Cysteine is glucogenic in the animal body, possibly through the intermediate formation of serine. On complete oxidation of cysteine and cystine, the sulfur is found in the urine either as inorganic sulfate or to some extent as organic esters of sulfuric acid with such compounds as indoxyl, phenol, etc. This is also discussed in Chapter 20.

ONE-CARBON INTERMEDIATES. In the discussion of the metabolism of glycine, serine, methionine, cystine, and histidine, mention is made of labile methyl groups and one-carbon intermediates. Whether these are

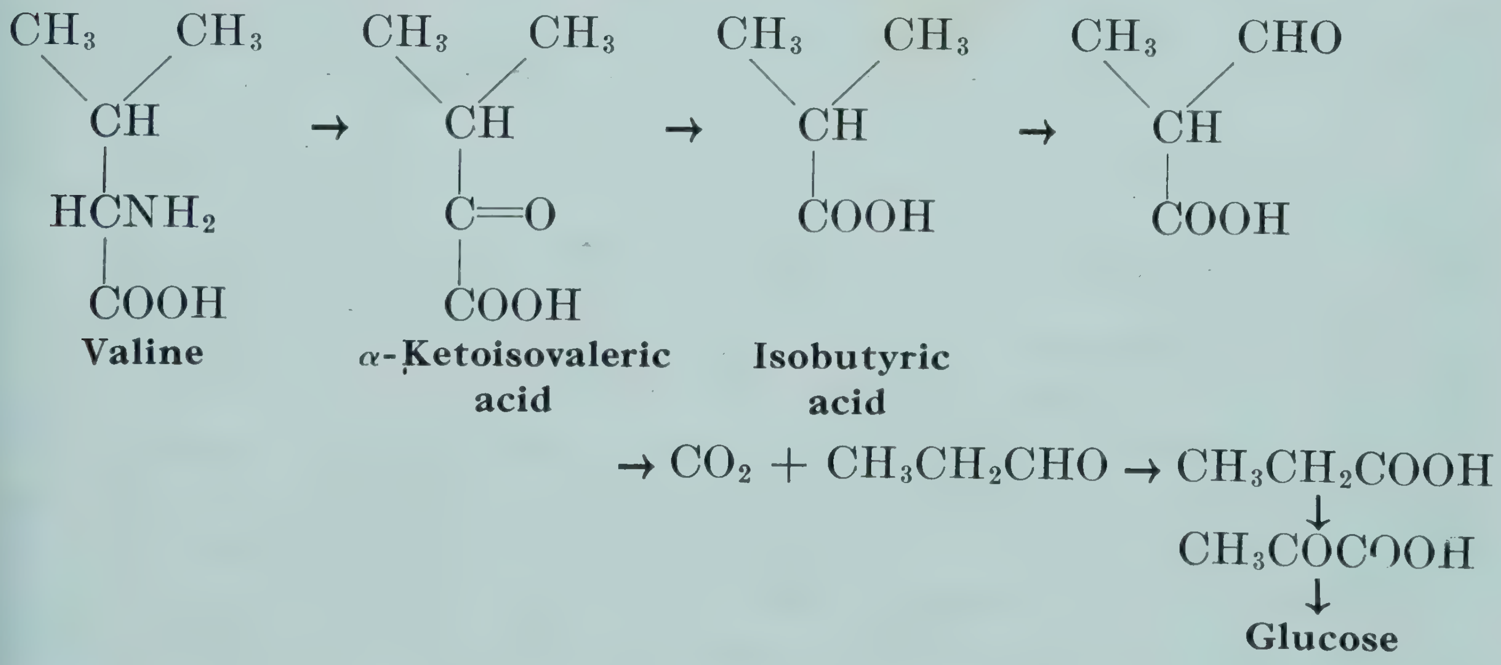

CH₂NH₂
COOH
CH₂OH
CHNH₂
COOH
CH₂OH
CH₂NH₂
CH₂OH
CH₂N⁺(CH₃)₃
COOH
CH₂N⁺(CH₃)₃
CH₃
S
CH₂
CH₂
CHNH₂
COOH
CH₃
NCH₂COOH
C=NH
NH₂
CH₃
C=O
CH₃
HCOOH
H
C
N
NH
HC
CCH₂CHCOOH
NH₂
N=COH
C
C—N
CH
N—C—N
R
CH₃NHCH₂COOH
N=COH
O=C
CCH₃
N—CH
R

the same compounds or not, it is known that they are metabolically related and for purposes of simplification have been formulated as HCOOH in the diagram on p. 1032 which illustrates some of the metabolic pathways of these one-carbon intermediates.

Leucine and Isoleucine. Relatively little is known concerning the metabolism of these two amino acids. They are not synthesizable from dietary constituents and are required in the diet for the growth of young animals and the maintenance of nitrogen equilibrium in the adult. Leucine and isoleucine are ketogenic rather than glucogenic amino acids. The conversion of leucine to acetoacetic acid possibly proceeds as follows:

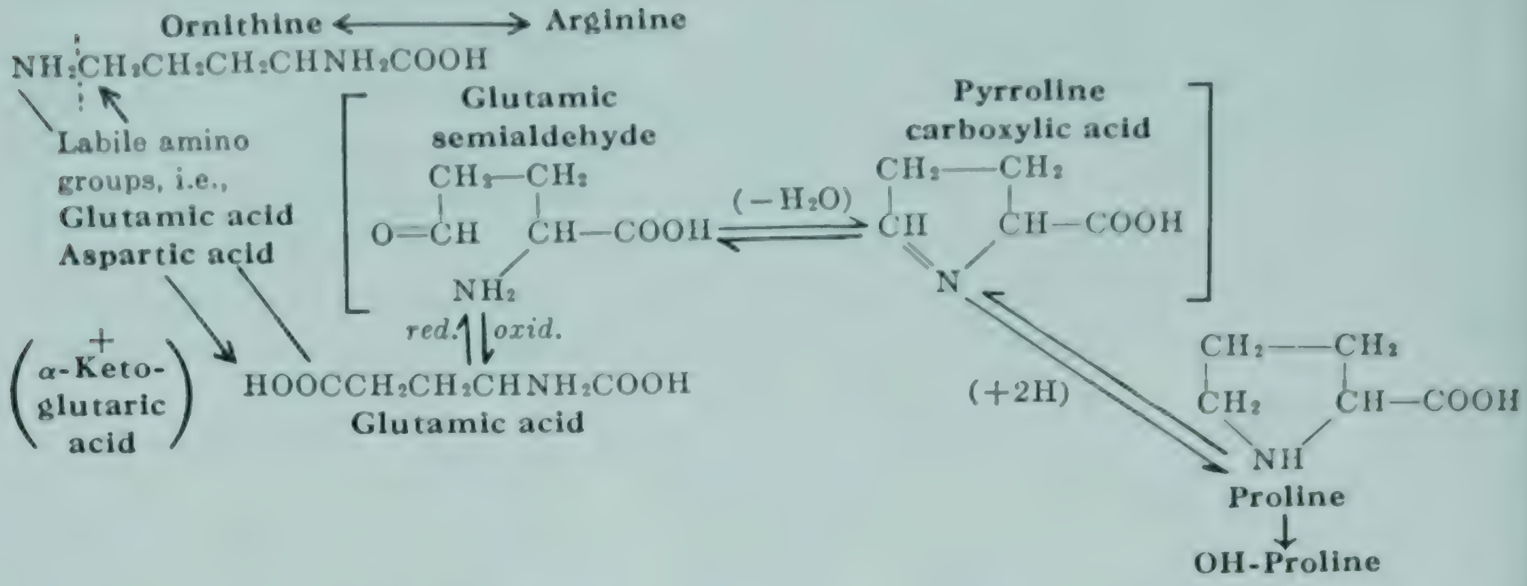


Valine. This amino acid is required in the diet in adequate amount, as evidenced both by growth studies with young animals and by the maintenance of nitrogen equilibrium in the adult human. A possible metabolic pathway involves deamination to form α-ketoisovaleric acid, since this compound will replace valine in an experimental diet.



Valine is convertible, in part at least, to glucose in the diabetic dog.

Proline and Hydroxyproline. These two amino acids can be synthesized by the animal body from dietary or tissue precursors, and proline is glucogenic in the diabetic dog. A probable precursor of proline is ornithine; since ornithine and proline are interconvertible in the organism, proline metabolism may proceed through ornithine formation. Glutamic acid has likewise been shown to arise from proline; further metabolism via this pathway would then be that of glutamic acid, and this may be the origin of the glucogenic action of proline. Hydroxyproline can be synthesized from dietary proline; there is some evidence that this reaction is not reversible, and that the further metabolism of hydroxyproline may not be similar to that of proline. These various relationships may be summarized as follows:

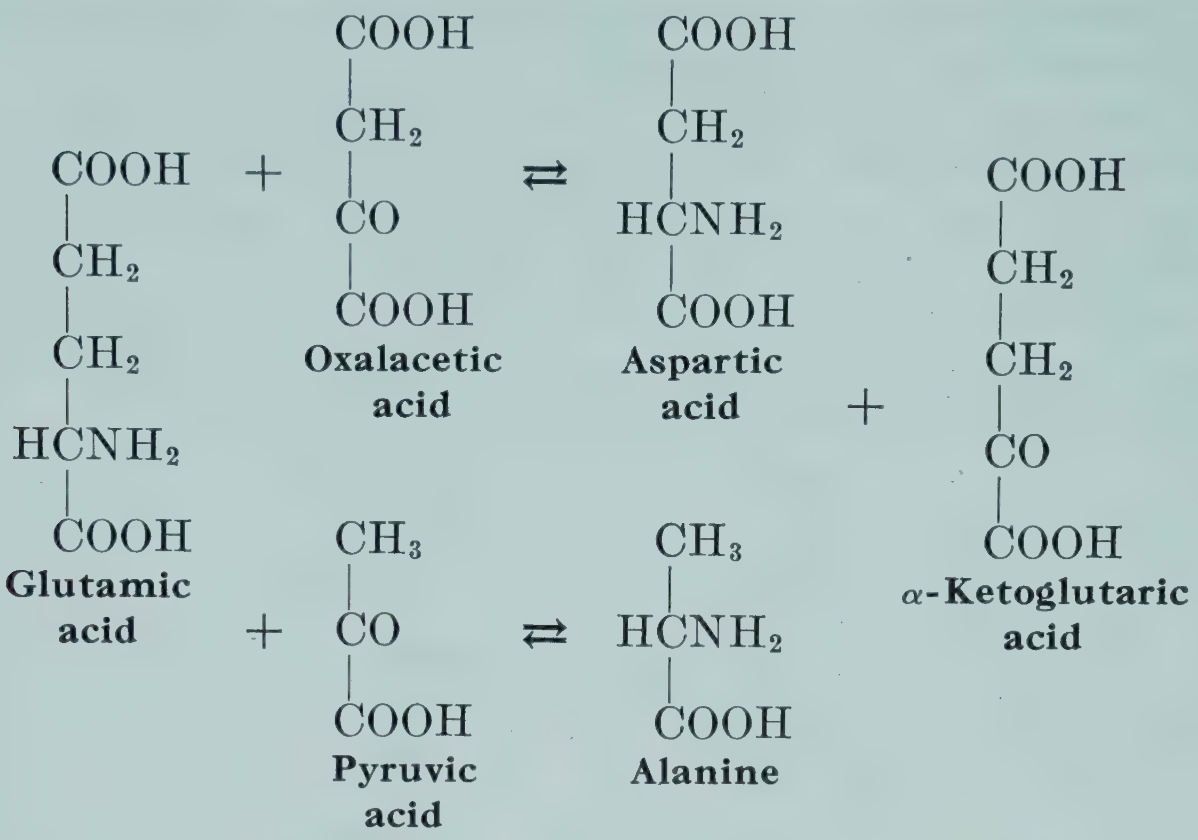

Ornithine ⟷ Arginine
NH₂CH₂CH₂CH₂CHNH₂COOH
Labile amino groups, i.e., Glutamic acid Aspartic acid
α-Keto-glutaric acid
HOOCCH₂CH₂CHNH₂COOH
Glutamic acid
red. oxid.
Glutamic semialdehyde
CH₂—CH₂
O=CH CH—COOH
NH₂
(−H₂O)
Pyrroline carboxylic acid
CH₂—CH₂
CH CH—COOH
N
(+2H)
CH₂—CH₂
CH₂ CH—COOH
NH
Proline
OH-Proline

Glutamic Acid. This dicarboxylic amino acid is present in fairly large amount in many animal and vegetable proteins. It is readily synthesizable by the animal body, and is thus dispensable from the diet; it is probable, however, that the ease of synthesis within the tissues is more properly a reflection of the important part played by glutamic acid in the general processes of nitrogen metabolism within the cell rather than an indication of a "nonessential" function.

Evidence that glutamic acid may be an important intermediate in general nitrogen metabolism is afforded by the work of Schoenheimer and associates using various amino acids labeled with isotopic nitrogen (N^{15}). When such labeled amino acids are fed to an animal, the isotopic nitrogen is found not only in the fed amino acid incorporated into the tissue proteins of the animal, but also to a significant extent in various other amino acids as well, and of these, glutamic acid usually exhibits the highest concentration of isotope. It is probable that transamination (see below) is partly responsible for this; the presence of glutamic acid in glutathione may also be connected with the apparently rapid transfer of dietary amino nitrogen via glutamic acid.

TRANSAMINATION. An important metabolic aspect of glutamic acid is its participation in the reactions of *transamination*. In the transamination reaction the amino group of glutamic acid is transferable to either pyruvic or oxalacetic acid to produce α-ketoglutaric acid and the amino acids alanine or aspartic acid, as the case may be. These reactions are reversible so that glutamic acid may be synthesized from α-ketoglutaric acid and either alanine or aspartic acid. The diagram on p. 1035 illustrates the transamination reactions.

Enzymes catalyzing the upper reaction (glutamic-aspartic transaminase) and the lower reaction (glutamic-alanine transaminase) have been isolated from animal tissues, and are believed to contain a pyridoxine (vitamin B_6) derivative as prosthetic group. A third postulated reaction, between aspartic acid and pyruvic acid to produce oxalacetic acid and alanine, is due, according to Green, to the presence of both of the transaminases mentioned. It is noteworthy that no free ammonia is formed during transamination, the reaction apparently involving condensation of amino acid and keto acid through the amino and keto groups to form an intermediate which is then split in such a way that the amino nitrogen is transferred from the original amino acid to the keto acid chain.



In addition to transamination, glutamic acid likewise undergoes reversible oxidative deamination.

$$\text{Glutamic acid} \rightleftarrows \alpha\text{-Ketoglutaric acid} + NH_3$$

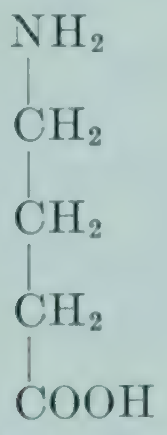
The reversal of this reaction is one of the few known examples of the biological synthesis of an amino acid from the keto acid and ammonia, and may represent one of the pathways for the demonstrated (although limited) conversion of dietary ammonia nitrogen into amino acid nitrogen.

As would be expected from the equilibrium between glutamic acid and its keto acid, α-ketoglutaric acid, which is an intermediate in carbohydrate metabolism (p. 990), glutamic acid is glucogenic in the diabetic dog. Certain other amino acids such as proline, ornithine, and histidine are known to give rise to glutamic acid in metabolism, and this may also explain their glucogenic action. The other keto acids involved in transamination (oxalacetic acid and pyruvic acid) are likewise carbohydrate breakdown products, and this fact may represent an important link between amino acid metabolism and carbohydrate metabolism.

Glutamic acid is combined as one of the three portions of the molecule of the vitamin pteroylglutamic (folic) acid, the other two being *p*-aminobenzoic acid and pteroic acid; the various conjugated forms of this vitamin are due to the multiplicity of glutamic acid units present in peptide linkage—e.g., the conjugate in liver contains seven such units while that obtained from fermentation contains three. (See Chapter 35.)

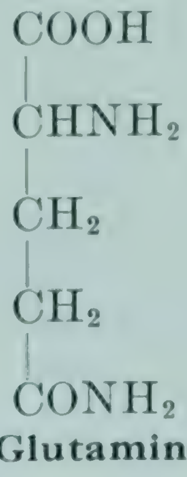
Glutamic acid appears to be present in proteins largely in the form of its amide, glutamine, $HOOC{\cdot}CH(NH_2){\cdot}CH_2{\cdot}CH_2{\cdot}CONH_2$. Free glutamine has been found in small amount in the blood, and according to Van Slyke and associates, glutamine or a glutaminelike compound is of importance as a precursor of urinary ammonia. Glutamine has also been implicated in the processes of urea formation by the liver, but its role here, if any, is obscure. The reported presence in cancer tissue of the "unnatural" optical isomer of glutamic acid, D-glutamic acid, has not been confirmed, but D-glutamic acid is found in the capsule of the anthrax bacillus and in other products of bacterial origin.

Glutamic acid is decarboxylated in the α position to give γ-aminobutyric acid. Glutamic decarboxylases are widespread in plants and bacteria, but have been found only in the higher portions of the central nervous system of animals. The importance of glutamic acid, glutamine, and γ-aminobutyric acid in the metabolism of the brain is not yet clear but the present evidence indicates that these compounds must play a basic part in the physiology of nervous tissue.



$$\begin{array}{l} NH_2 \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ COOH \end{array}$$

γ-Aminobutyric acid



$$\begin{array}{l} COOH \\ | \\ CHNH_2 \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CONH_2 \end{array}$$

Glutamine

Aspartic Acid. The metabolic aspects of this amino acid are less well understood than those of glutamic acid, but it appears to be similar to the latter in certain respects. It is readily synthesizable within the body, and shares with glutamic acid in the transfer of dietary amino nitrogen as described on p. 1034, but to a lesser extent. Synthesis probably proceeds via the transamination reaction involving the keto acid, oxalacetic acid, and glutamic acid as described previously. Metabolic breakdown by the reversal of this reaction to form oxalacetic acid, which is a carbohydrate intermediate, serves to explain the glucogenic power of aspartic acid as well as to indicate the pathway of further degradation. The amide of aspartic acid, asparagine, $HOOC{\cdot}CH(NH_2){\cdot}CH_2{\cdot}CONH_2$, is found in tissue proteins, but its significance is obscure.

Histidine. Histidine is required in the diet of the young growing animal but is apparently dispensable from the diet of the adult human, as established by the maintenance of nitrogen equilibrium in man on diets free of histidine. Here, as with arginine, it may be that rate of synthesis is the limiting factor, with the possibility that bacterial action in the intestinal tract may also be concerned. Histidine is apparently metabolized via the pathway shown at top of p. 1037.[75]

In addition to its presence in tissue proteins, histidine is found in the animal body in the muscle constituents carnosine (β-alanyl histidine) and anserine (β-alanyl methyl histidine), and it is probably a precursor of the red blood cell constituent ergothioneine, which is a betaine of thiolhistidine. Histidine is regularly found in the urine during pregnancy (and also

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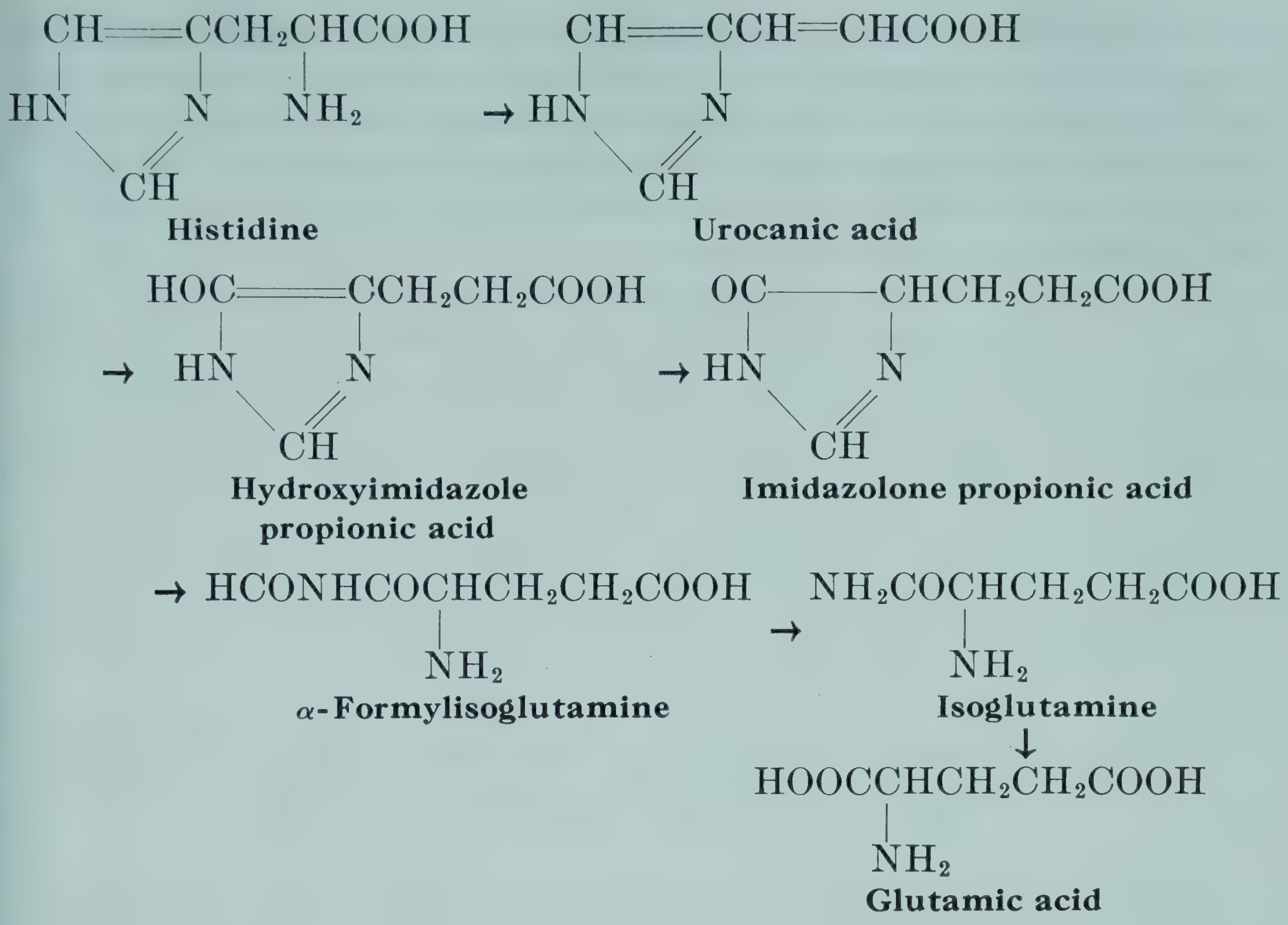
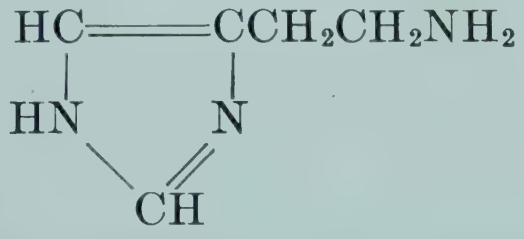
[75] Edlbacher and Kraus: *Z. physiol. Chem.*, **191**, 225 (1930).



in some other conditions); the significance of this is unknown. Histidine is glucogenic, possibly because it gives rise to glutamic acid during metabolism.

Decarboxylation of histidine produces histamine:

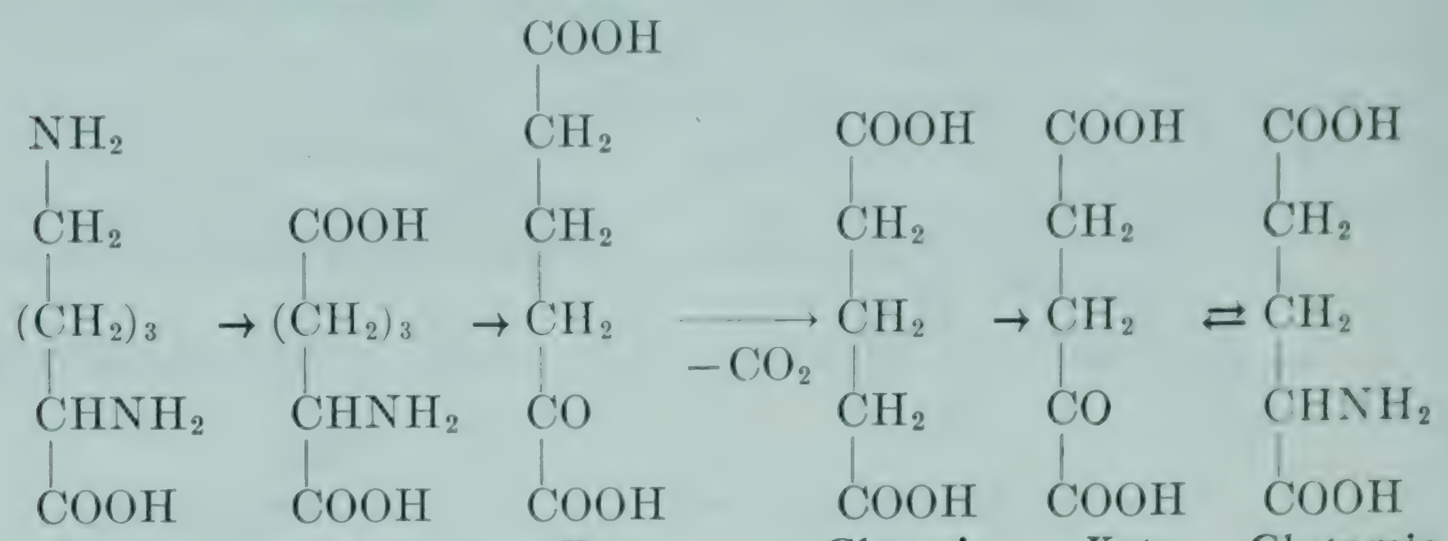


Histamine

Histamine is an organic base with a very powerful pharmacological action on certain body structures. In addition to its well-known effect on blood pressure (capillary dilatation), it is a potent stimulant of gastric secretion (see p. 360), and has been implicated in the complex symptomatology of anaphylactic shock and in eclampsia. Drugs which have an action specifically antagonistic to that of histamine are known as antihistamines; many if not all of these compounds are structural analogs of histamine and presumably owe their effect to an antimetabolite action (see Chapter 36).

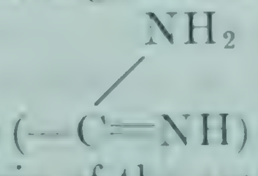
Lysine. This amino acid is required in the diet of the young growing animal and for the maintenance of nitrogen equilibrium in the adult human. It is not glucogenic in the diabetic dog. It is unique among all the amino acids thus far investigated in that it does not appear to be capable of obtaining its nitrogen from other dietary sources, as can be done by certain other amino acids (see discussion on p. 1022). Lysine, however, can contribute its nitrogen to other amino acids after metabolic breakdown.

The metabolism, therefore, may involve an oxidative deamination which for some reason is irreversible. This is borne out by the fact that neither the keto acid nor the hydroxy acid derivative of lysine can substitute for this amino acid in experimental diets. Studies have indicated that lysine may first form α-aminoadipic acid which is then converted into glutaric acid and hence to glutamic acid.



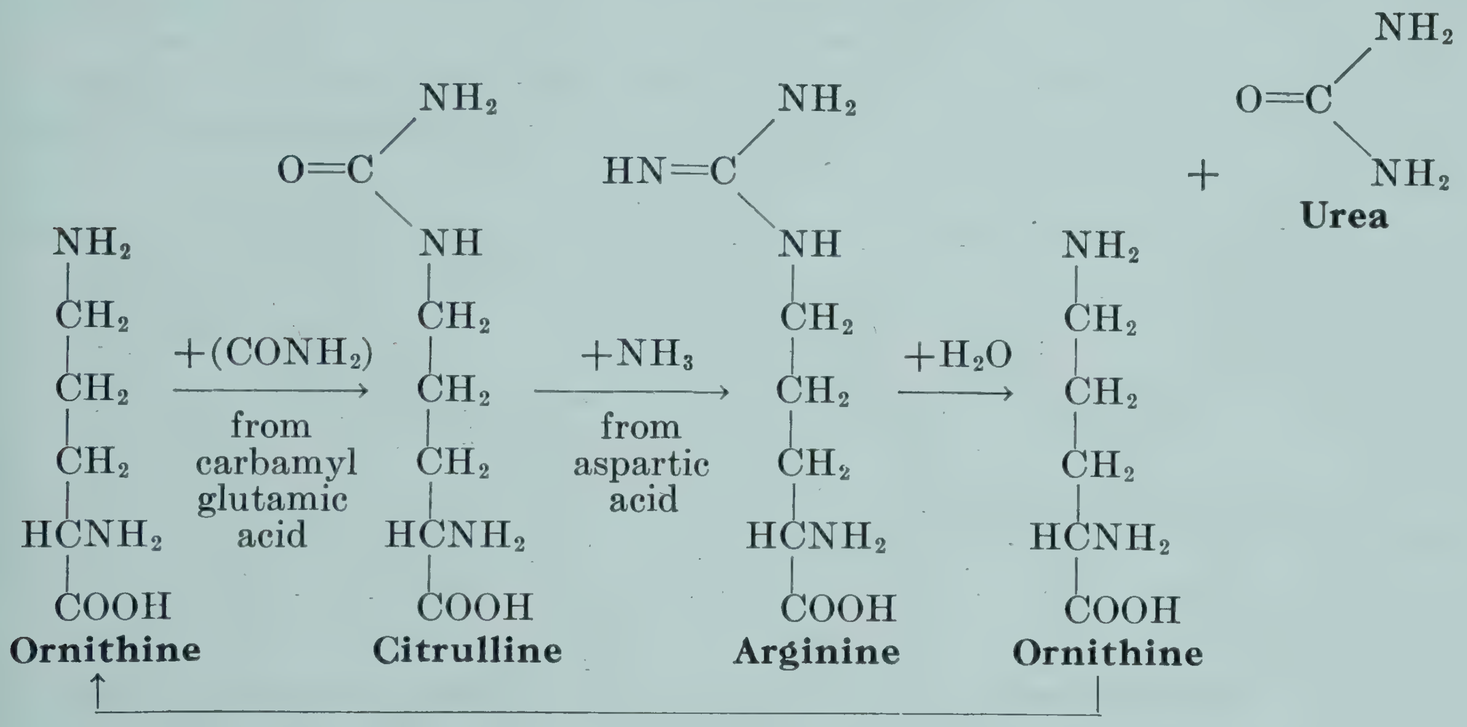
Lysine
α-Amino-adipic acid
α-Keto-adipic acid
Glutaric acid
α-Keto-glutaric acid
Glutamic acid

Arginine. The question of whether or not this amino acid can be synthesized by the animal body has been the subject of considerable debate in the past. It now appears to be settled, largely through the work of Rose, using diets containing mixtures of pure amino acids, that arginine can be synthesized by the tissues of the young growing rat, for example, but not at a rate fast enough to supply the needs of the animal for optimal growth. It is thus indispensable in this sense in the diet of the young growing rat. The adult rat (and the adult human) apparently can supply arginine by synthesis from other sources at a rate adequate for nutritional needs. On the other hand, the requirements of the chick for arginine are such that this amino acid must be considered indispensable in the diet—an example of species differences with respect to amino acid requirements.

Various studies using amino acids labeled with isotopic nitrogen and hydrogen have indicated that arginine is synthesized in the body by the addition of an amidine group  $(-\overset{NH_2}{C}=NH)$ to ornithine, $HOOC.CH(NH_2)\cdot CH_2\cdot CH_2\cdot CH_2\cdot NH_2$. The origin of the amidine group is not known (a possible mechanism is by the ornithine cycle discussed below), but ornithine is readily synthesizable biologically—for example, from proline (p. 1033). Evidence that the synthesis of arginine is reversible is not available, but it is known that the amidine portion may be transferred to glycine to form guanidoacetic acid in the synthesis of creatine (p. 1026). Arginine also may be degraded to form ornithine and urea in the presence of the enzyme arginase, as described below. There is evidence that arginine may give rise via ornithine to proline, hydroxyproline, and glutamic acid, and these may represent further stages in metabolism. Both arginine and ornithine are glucogenic, possibly because of their convertibility to the amino acids just mentioned. There is some evidence that the α-amino nitrogen of arginine does not undergo oxidative deamination to form the corresponding keto acid, or if so, that this process is irreversible.

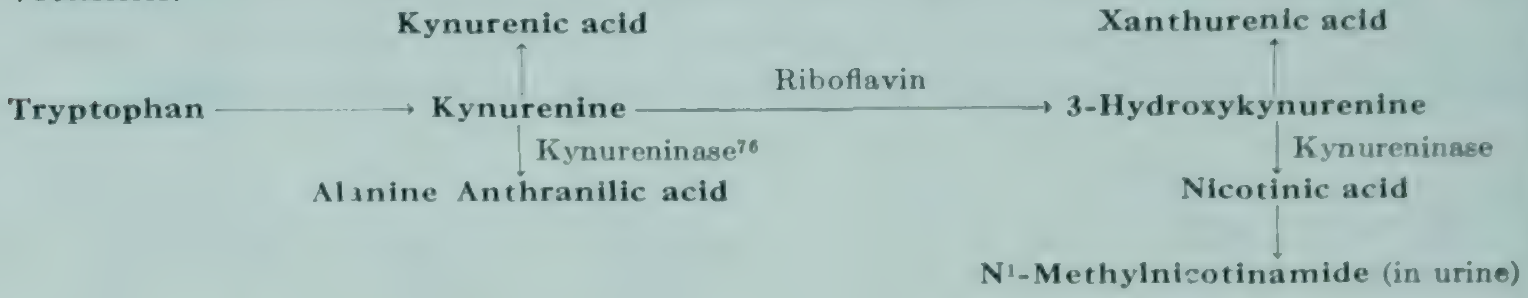
Interest in the metabolic function of arginine has been directed primarily toward its possible relation to the synthesis of creatine and the formation of urea. The role of arginine in creatine formation is described in detail on p. 1026. Interestingly enough, an arginine analog of phosphocreatine (namely, phosphoarginine) appears to serve in place of phosphocreatine in the muscles of certain lower animal species.

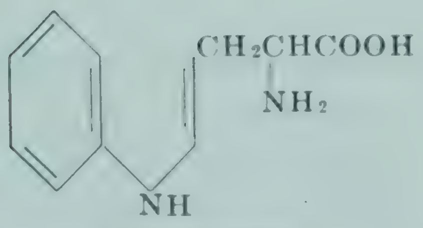
The possible role of arginine in urea formation by mammalian liver has been evident for a long time because of the presence of the enzyme arginase in liver; arginase catalyzes the hydrolytic splitting of arginine into ornithine and urea. According to Krebs and Henseleit, arginine enters into a cyclic mechanism, along with the amino acids ornithine and citrulline, for the conversion of ammonia into urea in the liver. On the basis of later developments, the process is now pictured as follows:



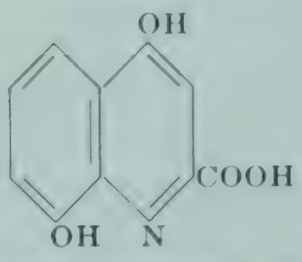
According to this view, ammonia and carbon dioxide are added to the amino acid ornithine to produce the amino acid citrulline. On the further addition of ammonia to citrulline, arginine is formed which then undergoes action by the enzyme arginase to produce urea and to regenerate ornithine, which can then proceed through the cycle again. The original evidence for this theory was based largely on the demonstration that both ornithine and citrulline catalyzed the synthesis of urea from free ammonia by liver slices. Additional evidence tending to support the theory has come from several sources. If the liver slices are incubated in the presence of bicarbonate containing isotopic carbon, the carbon isotope is found in the urea synthesized. Schoenheimer's work on the distribution of isotopic dietary nitrogen between the amidine moiety and the ornithine moiety of arginine isolated from tissue proteins, and the isotope content of the urinary urea, led him to conclude that the results supported the ornithine cycle theory. According to Gornall and Hunter, the accumulation of citrulline in liver tissue during urea formation under special conditions may be demonstrated, and citrulline may also be found in small amount in the blood. Beadle and Tatum also have presented evidence that the ornithine-cycle mechanism functions in the red bread mold *Neurospora*.

Tryptophan. This amino acid is required in the diet for growth of the young animal and the maintenance of nitrogen equilibrium in the adult human. It has several metabolic pathways which appear to vary somewhat in different species of animals. One of the most interesting findings in recent years is that tryptophan is converted in the body to the vitamin, nicotinic acid (niacin). Other end products appear to be alanine, anthranilic acid, kynurenic acid, etc. (cf. diagram). The quantitative aspects of these known metabolic pathways have not as yet been elucidated, but it appears that the conversion to nicotinic acid plays a minor role, for it requires from 50 to 300 g. of tryptophan to make 1 g. of the vitamin.

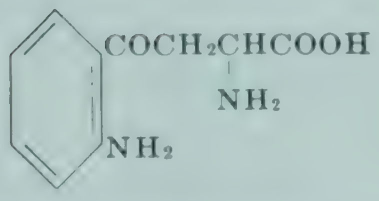

Kynurenic acid
Xanthurenic acid
Riboflavin
Tryptophan
Kynurenine
3-Hydroxykynurenine
Kynureninase[76]
Kynureninase
Alanine Anthranilic acid
Nicotinic acid
N1-Methylnicotinamide (in urine)


CH2CHCOOH
NH2
NH

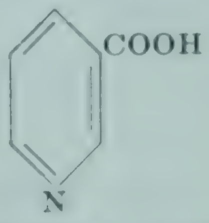
Tryptophan



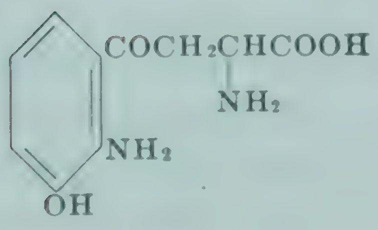
Xanthurenic Acid


COCH2CHCOOH
NH2
NH2

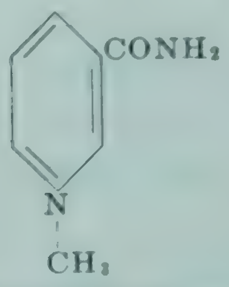
Kynurenine



Nicotinic Acid

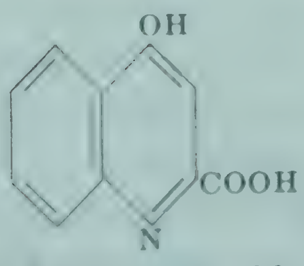

COCH2CHCOOH
NH2
NH2
OH

3-Hydroxykynurenine

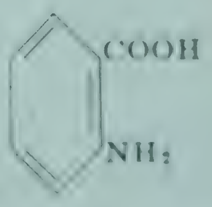


N^1-Methylnicotinamide

CH_3CHNH_2COOH
Alanine



Kynurenic Acid



Anthranilic Acid

Some animal species (but not others) will excrete kynurenic acid in the urine after the ingestion of large doses of tryptophan (Jackson). There is some basis for the belief that kynurenic acid is an end product and not an intermediate in tryptophan metabolism. Kynurenine is claimed to be a hormone for *Drosophila.* Tryptophan is converted into 4,8-dihydroxyquinoline-2-carboxylic acid (xanthurenic acid) by the rat on a diet deficient in vitamin B_6. The red bread mold *Neurospora* can synthesize tryptophan from indole and serine. The reverse of this reaction, i.e., the production of indole by bacterial action on tryptophan in the intestinal tract, is believed to be the origin of the indole, skatole (methyl indole) and indican of the feces and urine. Tryptophan deficiency produces a type of cataract in the rat, and certain evidence indicates a relation between anemia, tryptophan, and vitamin B_6 (pyridoxine).

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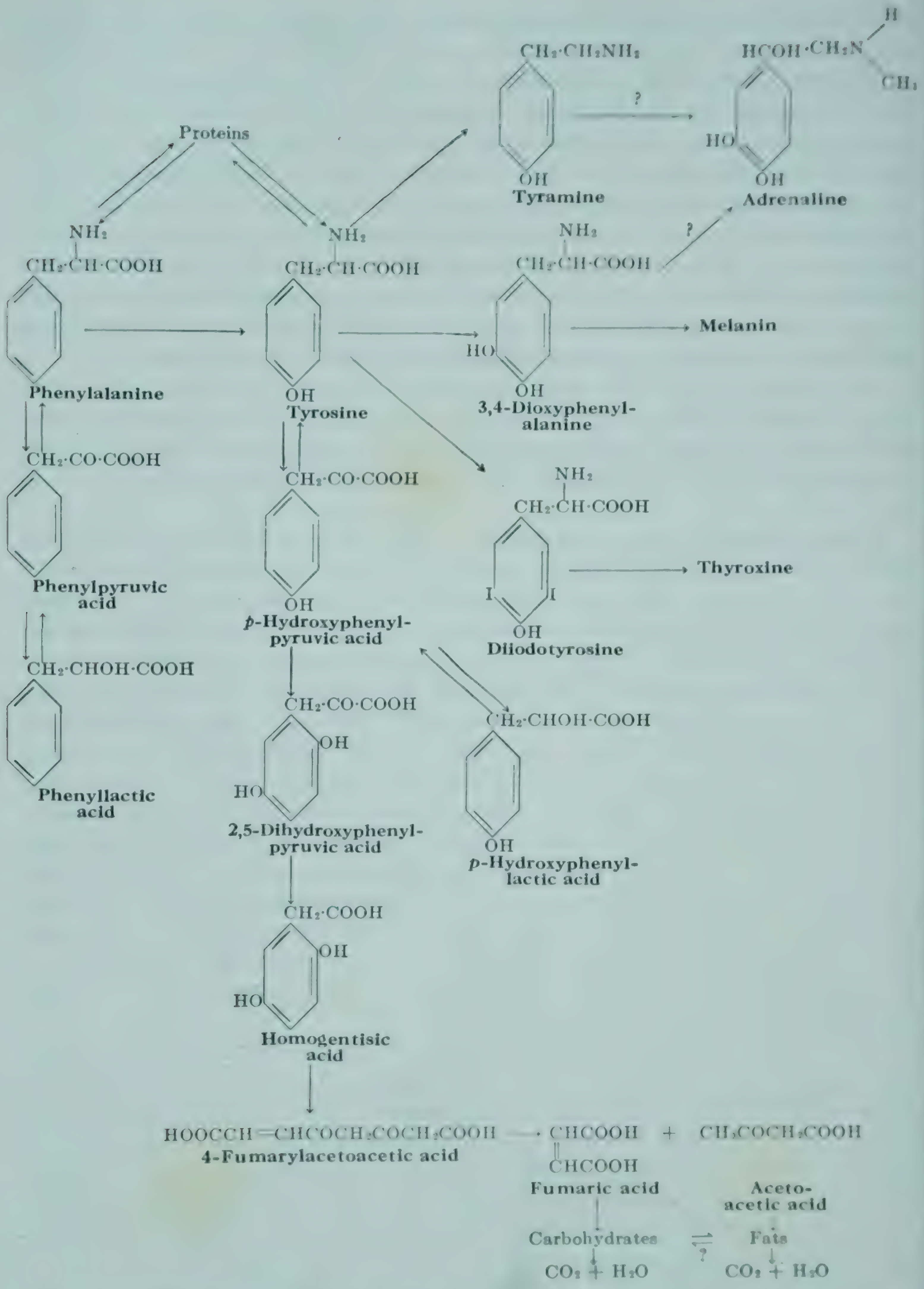
[76] Kynureninase contains pyridoxine (vitamin B_6) as a prosthetic group. In the absence of this vitamin there is an increased production of xanthurenic acid especially if riboflavin is present.

Tryptophan catabolism may also proceed by oxidative deamination to the corresponding keto acid, indolepyruvic acid, since this compound (and indolelactic acid) will replace tryptophan in experimental diets. Tryptophan is apparently not ketogenic and will alleviate experimental ketonuria in the rat.

Phenylalanine and Tyrosine. These two aromatic amino acids usually are considered together because of their close metabolic relationship. Of the two, phenylalanine cannot be synthesized by the animal body (except from its corresponding keto-or hydroxy-acid, which are not ordinary constituents of the diet) and therefore must be present in the diet in adequate amounts. The needs of the animal for tyrosine, however, can be readily supplied by dietary phenylalanine, as established by growth studies with young rats and by the maintenance of nitrogen equilibrium in the adult human. Phenylalanine, therefore, is convertible into tyrosine by the organism; this conversion has been directly demonstrated by the feeding of phenylalanine labeled with isotopic hydrogen (deuterium) to rats, followed by the subsequent isolation of tyrosine from the animal's tissue proteins; such isolated tyrosine contained sufficient deuterium to establish its origin from the fed phenylalanine. All available experimental evidence indicates, however, that the conversion of phenylalanine into tyrosine is *irreversible;* tyrosine cannot be converted into phenylalanine in the animal body.

Knowledge concerning the intermediary metabolism of both phenylalanine and tyrosine is still far from complete. In addition to their roles as essential components of the body proteins, these two amino acids, and particularly tyrosine, appear to be concerned with the formation of a variety of physiologically important substances such as thyroxine, adrenaline, the pigment melanin, and others. Certain of the known or postulated metabolic interrelationships of tyrosine and phenylalanine are summarized in the diagram on p. 1042.

Both phenylalanine and tyrosine are ketogenic amino acids, i.e., they can be shown to give rise to acetoacetic acid under the proper conditions, and neither amino acid is glucogenic. It is postulated, therefore, that after the loss of nitrogen which is excreted as urea, both phenylalanine and tyrosine are normally oxidized to carbon dioxide and water through the intermediate formation of acetoacetic acid. Whether these two amino acids follow a common metabolic pathway (i.e., phenylalanine → tyrosine


Proteins
CH₂·CH₂NH₂
OH
Tyramine
?
HCOH·CH₂N
H
CH₃
HO
OH
Adrenaline
NH₂
CH₂·CH·COOH
Phenylalanine
NH₂
CH₂·CH·COOH
OH
Tyrosine
NH₂
CH₂·CH·COOH
HO
OH
3,4-Dioxyphenyl-alanine
?
Melanin
CH₂·CO·COOH
Phenylpyruvic acid
CH₂·CHOH·COOH
Phenyllactic acid
CH₂·CO·COOH
OH
p-Hydroxyphenyl-pyruvic acid
NH₂
CH₂·CH·COOH
I
I
OH
Diiodotyrosine
Thyroxine
CH₂·CO·COOH
OH
HO
2,5-Dihydroxyphenyl-pyruvic acid
CH₂·CHOH·COOH
OH
p-Hydroxyphenyl-lactic acid
CH₂·COOH
OH
HO
Homogentisic acid
HOOCCH=CHCOCH₂COCH₂COOH
4-Fumarylacetoacetic acid
CHCOOH
CHCOOH
Fumaric acid
+
CH₃COCH₂COOH
Aceto-acetic acid
Carbohydrates
?
Fats
CO₂ + H₂O
CO₂ + H₂O

$\rightarrow$ intermediates $\rightarrow$ $CO_2 + H_2O$) is a matter of dispute. Possible direct metabolites of phenylalanine other than tyrosine include phenylpyruvic and phenyllactic acids; phenylpyruvic acid is found in the urine of animals after feeding with phenylalanine.

Fölling reported that a certain proportion of individuals with mental disease regularly excrete phenylpyruvic acid in the urine. This condition has been called *oligophrenia phenylpyruvica.* If phenylalanine is administered to individuals suffering from this metabolic abnormality, the excretion of phenylpyruvic acid is increased.[77] Thus there is good evidence that phenylpyruvic and phenyllactic acids are produced from phenylalanine; there is equally good evidence that the reactions leading to their production are reversible; for instance, both phenylpyruvic acid and phenyllactic acid can replace phenylalanine in the diet of the growing rat.

The metabolic fate of tyrosine is somewhat better understood than that of phenylalanine; the irreversible blockage between tyrosine and phenylalanine permits a more precise estimation of the significance of possible intermediates obtained under experimental and abnormal conditions. As with phenylalanine, possible metabolites of tyrosine include the corresponding keto- and hydroxy-acids, *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids, respectively. Both of these compounds have been isolated from human and animal urine under certain conditions. Medes has described a condition called *tyrosinosis* (only one such case has thus far been found) in which an adult human excreted significant amounts of *p*-hydroxyphenylpyruvic acid in the urine. The output of this compound was augmented with increased intake of protein or of pure tyrosine or phenylalanine; feeding the compound itself resulted in the unchanged excretion of most of it. Levine and associates discovered that the infant human may exhibit a spontaneous defect in tyrosine metabolism characterized by the excretion of significant amounts of *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids, in the presence of a low vitamin-C intake and an excessive intake of tyrosine or phenylalanine, either in the form of protein or as the pure amino acids. Vitamin-C administration abolished the defect except in the presence of excessive amounts of phenylalanine or tyrosine. Sealock and associates have shown that the scorbutic guinea pig excretes tyrosine metabolites (*p*-hydroxyphenylpyruvic acid, homogentisic acid) after administration of tyrosine or phenylalanine; vitamin-C administration prevented the excretion of these compounds. It has also been shown that scorbutic guinea-pig liver is unable to oxidize tyrosine *in vitro* except in the presence of vitamin C.

It would appear from these and other findings that the keto acid of tyrosine is a normal intermediate in tyrosine metabolism, and that vitamin C is concerned in the further utilization of the keto acid. The possibility remains open, however, that oxidative deamination of tyrosine is only one metabolic pathway and that the metabolism may follow some as yet unknown sequence of reactions.

Further knowledge concerning tyrosine metabolism has been afforded by the study of the metabolic abnormality known as *alkaptonuria.* In this relatively rare condition, individuals excrete the compound homogentisic acid (for structure see diagram on p. 1042) in the urine.[78] In the presence of alkali and oxygen, homogentisic acid forms a dark brown or black pigment, and it is the darkening of the urine under these conditions which usually leads to the discovery of alkaptonuria. Homogentisic acid is believed to be a normal intermediate in tyrosine metabolism, probably through the intermediary formation of 2,5-dihydroxyphenylpyruvic acid (see diagram, p. 1042), and the metabolic defect in alkaptonuria is thought to be the lack of ability to carry the oxidation beyond the stage of homogentisic acid formation. Feeding of tyrosine or phenylalanine to an alkaptonuric increases the output of homogentisic acid, and fed homogentisic acid is excreted unchanged. The normal individual is able to oxidize homogentisic acid completely. Alkaptonuria may be produced experimentally in animals (and in man) by prolonged or excessive feeding of either tyrosine or phenylalanine, and in scurvy. Vitamin-C administration will correct the alkaptonuria of the scorbutic guinea pig, but has no apparent effect on human alkaptonuria.

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[77] This disease is characterized by the fact that the individual has lost the ability to convert phenylalanine into tyrosine. As a consequence phenylalanine must be oxidized via the pathway phenylalanine → phenylpyruvic acid → phenylacetic acid. Phenylacetic acid is a neurotoxic agent which is detoxified by combination with glutamine to yield phenylacetylglutamine. As pointed out on p. 1036, glutamic acid and glutamine appear to be of special significance in the metabolism of the higher centers of the central nervous system. All patients with *oligophrenia phenylpyruvica* are mentally defective. Is this due to a chronic deficiency of glutamine?

Certain other aspects of tyrosine metabolism are of physiological importance. Both the diiodotyrosine and thyroxine of the thyroid gland are considered to arise from tyrosine. Both of these substances have been isolated from casein and other tyrosine-containing proteins treated *in vitro* with alkaline iodide solution, and the incubation of thyroid gland slices in the presence of radioactive iodide as a tracer results in the production of diiodotyrosine and thyroxine containing the radioactive material. Epinephrine is likewise thought to originate from tyrosine. It has been shown that animal tissue can decarboxylate tyrosine to yield tyramine; oxidation and methylation of this compound could produce epinephrine.[79] An alternate possible pathway would involve the intermediate formation of 3,4-dihydroxyphenylalanine, followed by decarboxylation to yield hydroxytyramine, and oxidation and methylation to produce epinephrine.

According to Bloch and to Raper, it would appear that the pigment melanin is related to tyrosine metabolism. Melanin is not a simple chemical substance, but rather a mixture of pigments of ill-defined composition. It is a normal skin pigment, and appears to be low or lacking in the condition known as *albinism*. It is produced in excessive amounts by melanotic tumor cells, and may even be excreted in the urine (*melanuria*) under these conditions. It is also produced in excessive amount in Addison's disease (bronzed diabetes). Bloch showed some years ago that the skin contains an enzyme (dopase) capable of converting the compound 3,4-dihydroxyphenylalanine (dopa) into melaninlike material, and that this enzyme was absent from the skin of albinos. According to Raper, the probable precursor of 3,4-dihydroxyphenylalanine is tyrosine.

I. GENERAL PROCEDURES IN METABOLIC STUDIES

1. Collection and Preservation of the Urine. In metabolism tests such as those given in this chapter accurate collection of urine for the *exact* 24-hour period is of the *utmost importance*. Proceed as follows: Empty the bladder at a given hour, e.g., 8 A.M., and *discard the urine*. Prepare a *thoroughly clean* bottle of proper size, introduce into it sufficient toluene to cover the bottom of the bottle, and use this bottle for the collection of all urine voided during the following 24 hours, including the urine obtained by emptying the bladder at the close of the 24-hour period, e.g., at 8 A.M. the next day. During the day, when not actually in use for the introduction of a urine fraction, the bottle should be kept in a refrigerator or cold room in order that the sample may not deteriorate before it is examined. Measure the volume of the sample and determine its specific gravity (see p. 785) and reaction before proceeding to the quantitative estimation of any specific urinary constituents.

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[78] See p. 810.

[79] See p. 1042.

For metabolism work on *dogs*, a cage is used which is provided with a screen bottom. Below this is a tray which slopes toward a central hole through which the urine passes into a bottle containing toluene. Each day's output is collected and filtered to remove hair, etc., and may then be diluted to a definite volume, usually 500 or 1000 ml. This procedure facilitates subsequent calculation.

To obtain the complete 24-hour secretion of urine of *dogs*, catheterization must be resorted to. Because of the difficulty of catheterizing male dogs, bitches (especially those who have had pups, and hence have stretched vaginas) are used for this type of experimentation. Care must be taken to have the catheter sterile, in order to avoid infection or cystitis. Rubber catheters are sterilized by boiling in water, while metal or glass catheters may be washed and kept in alcohol. The simplest procedure is to use a speculum (a nasal dilator is about the right size) to stretch the vagina and then to insert the catheter directly into the urethra, avoiding contact with the vaginal wall. The exact location of the urethral orifice can be determined with a little experience. Force should never be used. After the bladder has been emptied, it is washed out several times with warm water, the washings being collected with the urine and made up to volume as described above. Finally, if it does not interfere with the experiment, it is desirable to introduce about 50 ml. of warm saturated boric acid solution into the bladder from time to time.

The most satisfactory method for obtaining urine from a *rabbit* is to hold its head up between one's knees and to apply gentle pressure on the lower abdomen. By this process of "milking," practically all the urine may be obtained.

For the collection of urine in metabolism work on *rats*, a metabolism cage (see Appendix) is employed.

2. *Complete Analysis of Urine*. Ingest an ordinary mixed diet (or any special diet) and collect the urine accurately for a 24-hour period (see above). Measure the volume of the sample, determine the specific gravity, and preserve the urine (see above) until the following constituents have been determined (for methods of analysis, see Chapter 31): total solids, titratable acidity, hydrogen-ion concentration, total nitrogen, amino acid nitrogen, ammonia, urea, uric acid, creatinine, total sulfur, ethereal sulfates, inorganic sulfates, neutral sulfur (by difference), total phosphates and sodium chloride.

Calculate the nitrogen and sulfur partitions, i.e., the percentages of the total nitrogen and sulfur which occur in the different forms, and tabulate the data from the complete analysis. Compare your results with those listed in the tables on pp. 1055 and 1057.

3. *Collection and Preservation of Feces and the Mixing and Weighing for Analysis*. The old methods in vogue in metabolism work embraced the analysis of *dried* feces. Various investigators later demonstrated that the drying of feces was accompanied by losses and changes of some of the organic constituents of the feces. Therefore the chemical examination of all stools wherever possible should be made on the *fresh* feces. If a study is being made which extends over several days and it is desired to economize time and effort in the chemical examination, the daily fecal output or an aliquot portion of each stool may be collected in a friction-top can or pail of suitable size and preserved by thymol and refrigeration. This method has been found satisfactory when the feces are to be examined for inorganic constituents or total nitrogen. For the determination of fat, carbohydrate, etc., the *fresh* stool should be employed because of the possibility of error due to hydrolysis of fat to fatty acid, etc.

In the preservation of feces for the determination of total nitrogen, the following simple procedure may be used: Introduce each stool into a weighed friction-top can or pail and place the vessel in a cold room or refrigerator preferably near or below 0° C. At the end of the period, mix the feces thoroughly and analyze weighed portions. In case individual stools are analyzed, the stool should be collected in a *weighed* flat-bottomed porcelain dish.[80] After mixing the feces *very thoroughly*, the weight of dish, spatula, and feces is determined and the weight of the feces secured by difference.[81] A portion of the well-mixed feces is then introduced into a large weighing bottle containing a glass hoe. Desired amounts of feces are then removed for analysis and the exact weight of such amounts obtained by difference.

The daily output of feces in *dogs* is quite variable, so that in metabolism work it is advisable to collect feces in periods, rather than each day. The stools are collected from the screen floor of the cage and placed in weighed pans. They are dried by adding alcohol, stirring and evaporating on the water bath, and then weighed. A few drops of sulfuric acid should be added to prevent the loss of ammonia. After drying the feces may be ground in a mill or mortar, to facilitate uniform sampling.

4. *Separation of Feces.*[82] In order to differentiate the feces which correspond to the food ingested during any given interval, it is customary to cause the subject under observation to ingest some substance, at the beginning and end of the period in question, which shall sufficiently differ in color and consistency from the surrounding feces as to render such differentiation comparatively easy. Two markers very widely used in such tests are *wood charcoal* and *carmine*. In making an actual separation of feces in a metabolism experiment, proceed as follows: Just preceding or in the early part of the first meal (usually breakfast) of the metabolism test, ingest a gelatine capsule (No. 00) containing 0.2 to 0.3 g. carmine or charcoal. From this time collect all stools in *flat-bottomed* porcelain dishes and examine for the presence of the marker. All fecal matter containing portions of the marker may be considered as representing the diet in question. This fecal matter should be retained and preserved (see above). Just before or in the early part of the first meal (usually breakfast) following the end of the metabolism test, a second marker in a gelatin capsule should be ingested. The feces should be carefully inspected until the marker makes its appearance. Retain all fecal matter uncolored by the marker, and reject the remainder. Frequent difficulties are encountered in the practical separation of feces, but the character of such difficulties will be most satisfactorily impressed by the performance of actual separations. Chromic oxide (Cr_2O_3) has also been used as marker in feces separation. See Chapter 21.

5. *The Use of Agar-agar to Increase Fecal Bulk.* The indigestible hemicellulose agar-agar (as well as other gummy substances such as psyllium seed, bassorin, etc.) has the property of absorbing water readily and therefore when ingested it increases the bulk of the feces considerably. This fact is made use of in the determination of metabolic product nitrogen (see Exp. 11, p. 1055), and clinically in some forms of constipation.

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[80] The spatula for mixing the feces should be weighed with the dish.

[81] In case it is desired an *aliquot part* of each stool may be placed in a friction-top can or pail and preserved as a composite sample for the period.

[82] For other practical work on feces, see p. 452.

Experiment. **Ingest a uniform diet for four days, or longer for greater accuracy. Divide the interval into two equal periods and separate the feces by charcoal or carmine (see Exp. 4 above). During the second period ingest 10 g. of agar-agar at each meal. Collect the feces for each period (see Exp. 4, above) and note the increase in the daily excretion under the influence of the agar ingestion. What was the increase per gram of agar?**

II. EXPERIMENTS ON NITROGEN METABOLISM

1. Time Relations of Protein Metabolism. It is a well-known physiological fact that an interval elapses between the ingestion of protein food and the appearance in the urine of certain products representing the complete catabolism of this food. The chief among these is urea. The term *nitrogen lag* has been used to designate the period elapsing between the ingestion of protein and the excretion in the urine of a quantity of nitrogen equivalent to that contained in the protein.

Experiment. **Ingest a simple uniform diet whose exact composition has been determined by analysis or whose approximate composition has been estimated. (See Appendix for table showing composition of foods.) Continue this diet from 1 to 4 days. Collect the urine in 2-hour periods from 7 A.M. to 11 P.M. and in an 8-hour period between 11 P.M. and 7 A.M. Analyze each specimen for total nitrogen or urea. At the end of this preliminary period add to the uniform diet, at one meal, a weighed quantity (150–250 g.) of lean meat specially prepared and analyzed. Collect the urine in periods as before and determine total nitrogen or urea. Calculate the total nitrogen or urea excretion; tabulate the data and plot curves showing the course of the nitrogen excretion on the various days of the experiment. How long was the "nitrogen lag"?**

A less accurate experiment than the above but one which yields interesting data may be carried out as follows:

Ingest a simple diet whose nitrogen content can be estimated with some degree of accuracy. Collect the urine in 2-hour periods from 7 A.M. to 11 .M. and in an 8-hour period from 11 P.M. to 7 A.M. and analyze for total nitrogen or urea. The next day ingest the same diet plus 150–250 g. of lean meat whose nitrogen content has been determined by analysis or estimated. Collect the urine as upon the previous day and determine its total nitrogen or urea content. Plot curves showing the course of the nitrogen or urea excretion on each of the days. How soon after the ingestion of the large quantity of meat did you note an increase in the nitrogen or urea excretion? How many hours after the meal was the maximum quantity of nitrogen or urea excreted?

2. Digestibility and Biological Value of Protein. The term *protein utilization* or *biological value* is used to indicate the percentage of the ingested food nitrogen actually assimilated. *Absorbability* or *digestibility* indicates the percentage of the ingested food nitrogen which is absorbed.

The digestibility of food protein may be approximated by the following procedure. Ingest any diet of known nitrogen content for a period of three days (see Appendix, "Composition of Foods"). Longer periods are necessary for accurate work. Collect all feces from the diet, making the separations as directed on p. 1046, using carmine as the initial marker and charcoal as the final marker or vice versa. Preserve the feces as directed on p. 1045. Mix the total feces thoroughly and determine the nitrogen by the Kjeldahl method. The approximate nitrogen utilization may be calculated as follows:

$$\frac{(\text{Food N} - \text{Feces N}) \times 100}{\text{Food N}} = \text{Approximate percentage N utilization}$$

Inasmuch as the nitrogen content of the feces does not originate entirely from the food, but represents in part residual metabolic products, i.e., intestinal epithelium, bacteria, secretions, etc., a correction is usually made for metabolic nitrogen in more exact work (see p. 1055). The value thus obtained is more properly designated as the *percentage digestibility* or the *coefficient of digestibility.*

To determine the true percentage digestibility of food protein, proceed as follows: Ingest a nonnitrogenous diet as described on p. 1055 for a period of two days, using sufficient agar-agar to insure a daily fecal output which shall approximate in weight that obtained when the regular protein diet was ingested.[83] Separate and preserve the feces as directed on p. 1046. Mix thoroughly and analyze for nitrogen according to the Kjeldahl method. This gives the metabolic fecal nitrogen. Follow this period with a test period during which the protein in question is included in the diet. Calculate the percentage digestibility of the protein of the diet as follows:

$$\frac{[\text{Food N} - (\text{Fecal N} - \text{Metabolic N})] \times 100}{\text{Food N}} = \text{Percentage digestibility}$$

The estimation of protein utilization in the body must take into account not only absorption from the intestine, but also that fraction of the absorbed nitrogen which is retained.

Mitchell,[84] who has used this procedure extensively in the biological evaluation of proteins, felt that this could be done by determining the urinary nitrogen excretion on a nonnitrogenous diet (endogenous nitrogen) and subtracting this from the urinary nitrogen on the protein-containing diet, to obtain the urinary nitrogen actually due to the metabolism of the protein. It is not clear that this procedure is entirely valid, based as it is upon Folin's classical distinction between endogenous and exogenous metabolism, which now appears incorrect (see discussion on p. 1021). Nevertheless the procedure under the conditions prescribed by Mitchell is capable of giving reasonable and consistent results, whatever may be its theoretical basis. The nitrogen balance method has been employed by Rose to determine the indispensable amino acid requirements of young men.

Various investigators[84a] have pointed out that to determine the minimum amount of dietary protein necessary to maintain nitrogen equilibrium, data are required for at least two levels of nitrogen intake near the region of balance, in order that the value at exact equilibrium may be obtained by interpolation or extrapolation.

***Procedure.* Ingest a nonnitrogenous diet as described above, insuring sufficient energy to provide for the body's requirements so that oxidation of tissue for this purpose may be avoided. Feces and urine are collected as described in the early part of this chapter. By means of Kjeldahl analyses, values are determined for metabolic nitrogen of feces and endogenous nitrogen of the urine. Follow this period with a test period during which the protein under investigation is included in the experimental diet. The following**

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[83] It is frequently difficult to so regulate the agar-agar intake as to secure the proper fecal output. In such an event the proper value for metabolic nitrogen must be obtained by calculation. For example, if 89.1 g. feces were excreted per day on the protein diet, and 166.5 g. per day (with a nitrogen value of 0.5 g.) when agar was employed, the actual value for metabolic product nitrogen may be obtained by the following proportion, assuming that the content of metabolic nitrogen is proportional to the weight of feces excreted $89.1:166.5::x:0.5$. $x = 0.268$ g. metabolic nitrogen per day.

[84] Mitchell: *J. Biol. Chem.*, **58**, 873 (1924); *Ind. Eng. Chem., Anal. Ed.*, **16**, 696 (1944); *Nutrition Revs.*, **10**, 33 (1952).

[84a] Melnick and Cowgill: *J. Nutrition*, **13**, 401 (1937); Allison: *Adv. in. Protein Chem.*, **5**, 155 (1949)—a review of methods for the biological evaluation of proteins; Arnold and Schad: *J. Nutrition*, **48**, 377 (1952).

equation represents the percentage of the absorbed food nitrogen which is retained by the body:

$$\frac{[\text{Food N} - (\text{Fecal N} - \text{Metabolic N}) - (\text{Urinary N} - \text{Endogenous N})]100}{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})}$$

$$= \text{Biological Value}$$

3. Protein-sparing Action of Carbohydrate and Fat. The nonnitrogenous nutrients, carbohydrate and fat, have the power to diminish the extent of the catabolism of protein in the normal human body. In other words, they are said to "spare" protein. This point is illustrated in data reported by Von Noorden and Dieters, which are tabulated below.

PROTEIN-SPARING ACTION OF CARBOHYDRATE

Nitrogen Ingested	*Nitrogen in Urine*	*Remarks*
12.6 g.	10.4 g.	
12.6 g. + 200 g. sucrose	9.0 g.	13 per cent reduction in protein catabolism

It will be observed that the addition of 200 g. of sucrose to the diet was accompanied by a decrease of 13 per cent in the amount of protein catabolized. It has been established that carbohydrates are more efficient protein sparers than are the fats. For example, Voit found carbohydrate to produce a 9 per cent decrease in protein catabolism whereas fats produced only a 7 per cent decrease.

***Experiment.* Ingest a uniform diet of known or estimated nitrogen content for a period of four days. Collect and preserve the urine accurately (see p. 1044) in 24-hour samples and analyze the excretion of the *third and fourth days* for total nitrogen. On the fifth day *add 200 g. of sucrose to the diet.* Analyze this urine also for total nitrogen. Calculate your results and tabulate the data as shown in the table above.**

Did the sucrose influence the catabolism of protein in your body?

4. Influence of a High Caloric Nonnitrogenous Diet. When an individual fasts, a certain amount of protein tissue is consumed each day of the fast. The destruction of such tissue is rather low on the first day because the glycogen stores of the body are being utilized to furnish the necessary energy. If an individual, instead of fasting, ingests a diet of high calorific value and very low in nitrogen the output of nitrogen in the urine of the third or fourth day will be less than on the third or fourth day in fasting. This is due to the fact that the body derives sufficient energy from the high-calorie diet and there is less destruction of tissue protein than occurs in fasting. For a discussion of energy value of foods see "Determination of Fuel Value of Food" below, and the table "Composition of Foods," in the Appendix.

***Experiment.* Ingest a high-calorie diet which is very low in nitrogen or actually nonnitrogenous. A satisfactory diet may include sugar, butter, starch, cream, agar-agar, and water. (For energy values see below and table, Appendix.) Ingest such a diet for three days. Collect the urine in 24-hour periods,**

preserve, and analyze it for total nitrogen, acidity, and ammonia. Note the low nitrogen excretion on the third day as compared with the nitrogen output of the third day of fasting. If so desired, you may (at some later date) fast for three days and repeat the above analyses for comparison.

Determination of Fuel Value of Food. When organic substances are oxidized or burned in the human body, they liberate a certain amount of heat. This calorific energy or heat value varies according to the type of organic matter undergoing oxidation. Thus the proteins, fats, and carbohydrates of the diet when they are burned in the body yield different quantities of heat per unit of substance than do organic acids, alcohol, etc. The energy values of food protein, fat, and carbohydrate are as follows:

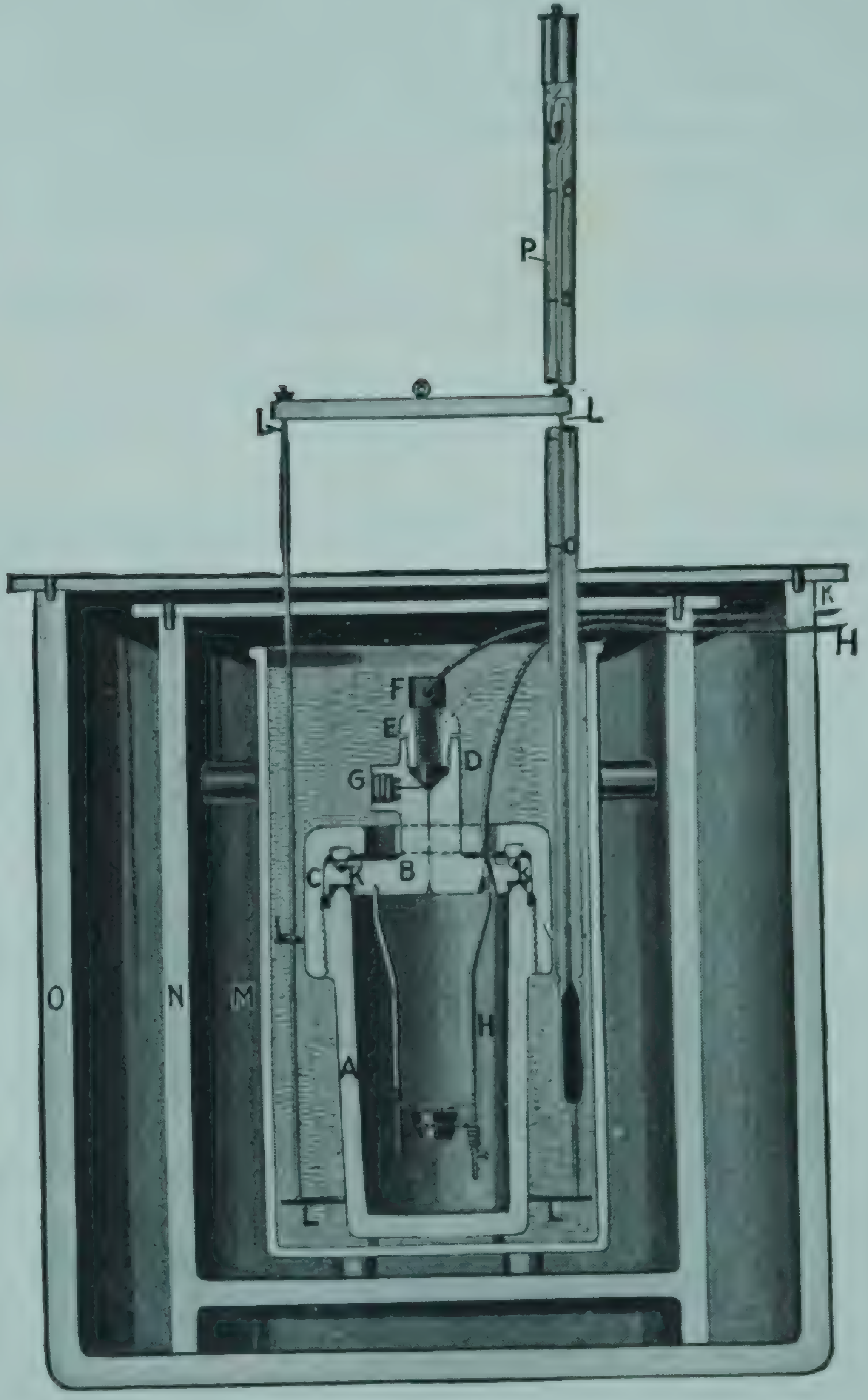

FIG. 260. BERTHELOT-ATWATER BOMB CALORIMETER.

Protein = 4.1 large Calories per g.
Fat = 9.3 large Calories per g.
Carbohydrate = 4.1 large Calories per g.

To allow for the incomplete digestion of these nutrients these caloric conversion factors are multiplied by the coefficients of digestibility and thus rounded off to 4, 9, and 4, respectively. The calorific value of foods may be estimated by applying these figures to the percentage composition as determined by chemical analysis. For this purpose, the proximate analysis includes moisture, ash, protein (N × 6.25), fat, crude fiber, and carbohydrate, the latter usually estimated by difference. Crude fiber represents the insoluble, indigestible residue remaining after alternate extractions with acid and alkali, and consists chiefly of cellulose, etc., from cell walls. Such calculations are predicated on the assumption of complete availability of the carbohydrates as estimated by difference, on the universal applicability of the factor 6.25 for converting total nitrogen to available protein, and on the assumption that the ether extract consists principally of glycerides.

In arriving at the total energy value of any given diet it is possible to burn weighed samples of the various foods in an oxygen atmosphere in an apparatus called a bomb calorimeter, illustrated in Fig. 260. By this means we may determine how much heat is liberated when the ingested food is oxidized in the body. A correction must be made for the incompletely oxidized substances of the urine and feces, e.g., organic nitrogen compounds. Thus while proteins yield about 5.7 Calories per gram when burned in a calorimeter, correction for incompletely oxidized urea and other N compounds reduces this value in the body to 4.1. Further correction must be made for indigestible carbohydrates usually grouped together under the designation "crude fiber." There is serious question, however, whether the latter, which resists hot acid and alkaline digestion, is an accurate measure of the polysaccharides which *in vivo* resist enzymatic digestion.

A simpler and less expensive form of apparatus for determining this calorific value of foods is the oxycalorimeter of Benedict and Fox, in which the volume of oxygen required to burn a known weight of substance is determined. A large mass of data concerning the heat value of foods has been collected and tabulated, and it is therefore possible to arrive at an approximate idea of the energy value of a diet by calculation (see table, Appendix).

5. *Influence of Purine-free and High Purine Diets.* The uric acid of the body has a twofold origin, i.e., it may arise from the metabolism of the purine (nucleoprotein, nucleotide) material of body tissue (glandular organs in particular) or it may arise from the ingestion of purine material. That uric acid which arises from the first source is called *endogenous* while that which arises from the second source is termed *exogenous.* Modern concepts hold that this distinction, made many years ago by Folin, is still valid. Secretory activity may also act to increase the endogenous uric acid output. The urine will therefore contain uric acid even though no precursor of the acid is ingested. We may also increase the uric acid output markedly by ingesting a high purine diet. However, no matter how much purine material is eaten, only a small part of it reappears in the urine as uric acid. That is, there must be some significant metabolic pathway for purine nitrogen other than excretion as uric acid. In gout there is an accumulation of uric acid in the blood. In this disease the excretion of uric acid may be low before an attack and increase considerably during an attack. The excretion of exogenous uric acid in gout is also much slower than normal.

Experiment. **Ingest a purine-free diet containing about 16 g. of nitrogen and consisting of egg, cheese, milk, starch, fruit, sugar, and water for a period of two days (for purine content of foods, see table, p. 1053). Determine or estimate the nitrogen content (see Appendix) and during the next two days substitute sweetbreads, thymus, or liver for all the nitrogen of the diet, maintaining the calorific value of the diet the same as before. Return to the original purine-free diet for a third interval of two days. During the final**

period of two days feed a diet of sweetbreads or liver containing 50 per cent more nitrogen than that of the first sweetbread period. Collect the urine for each of the eight days of the experiment and determine uric acid and total nitrogen or urea. Note the rise in the uric acid output during the sweetbread periods. The uric acid output on the purine-free diet is endogenous in origin. Tabulate your results. The data shown above were secured by Taylor and Rose in a similar but much more carefully controlled test than that just outlined.

INFLUENCE OF PURINE-FREE AND HIGH PURINE DIETS
NITROGEN INGESTION 10 G. DAILY (TAYLOR AND ROSE)

Urinary Constituents Determined (g. per day)	*Purine-free Diet*	*Purine Diet (Medium)*	*Purine Diet (Increased)*	*Purine-free Diet*
Uric acid N	0.09	0.14	0.24	0.07
Total nitrogen	8.9	8.7	9.1	8.8
Urea N (+NH_3)	7.3	7.1	7.1	7.05
Creatinine	1.57	1.49	1.51	

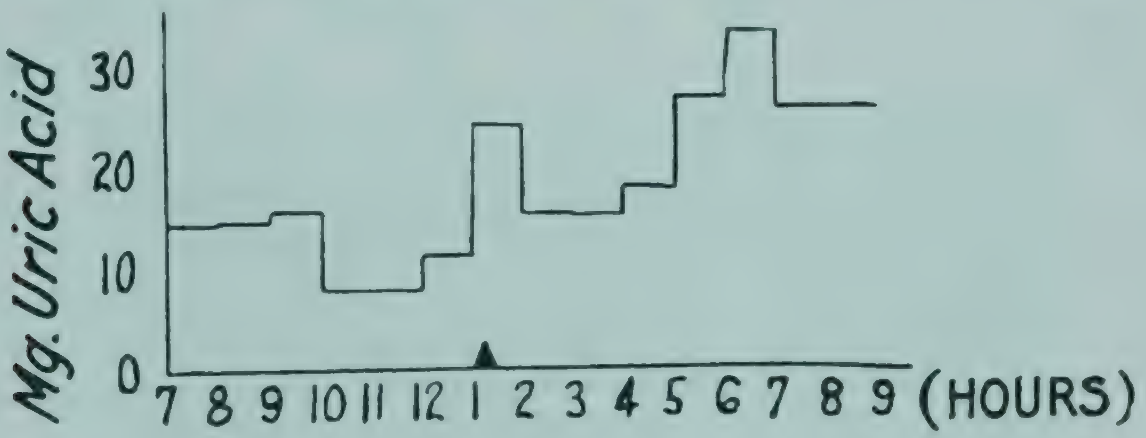

FIG. 261. INFLUENCE OF PROTEIN INGESTION ON ENDOGENOUS URIC ACID OUTPUT. GLUTEN (130 G.) INGESTED AT 1 P.M.
Courtesy, Mendel and Stehle: *J. Biol. Chem.*, **22**, 215 (1915).

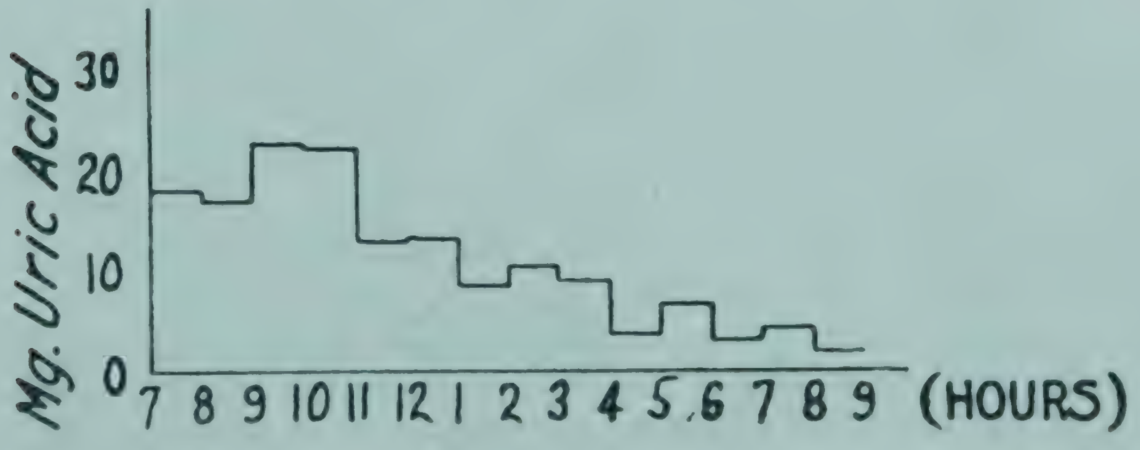

FIG. 262. ENDOGENOUS URIC ACID OUTPUT DURING FASTING.
Courtesy, Mendel and Stehle: *J. Biol. Chem.*, **22**, 215 (1915).

6. A Study of Endogenous Uric Acid Output. The uric acid in the urine originates from the purine material of the tissues (*endogenous*) and from the purine material ingested (*exogenous*). Mares claims that foodstuffs act to increase the endogenous uric acid output by stimulating the digestive glands to activity. A similar finding is reported by Mendel and Stehle. The foodstuff having the most pronounced influence was protein. *Pilocarpine*, which stimulates the digestive glands, was found to increase the endogenous uric acid output whereas *atropine* which inhibits secretory activity was found to decrease the output of endogenous uric acid. The influence of protein upon the endogenous uric acid excretion is shown by the chart in Fig. 261. The fasting output by the same individual is shown, for comparison, in Fig. 262.

PURINE CONTENT OF FOODS
(AFTER BESSON AND SCHMID)

Food	*Purine Nitrogen*
	per cent
Meats:	
Beef	0.037
Veal	0.038
Mutton	0.026
Pork	0.041
Liver	0.093
Tongue	0.055
Sweetbreads	0.330
Brains	0.028
Fowl:	
Chicken	0.029
Goose	0.033
Squab	0.058
Fish:	
Cod	0.038
Salmon	0.024
Herring	0.069
Pike	0.047
Trout	0.056
Sardines	0.118
Anchovies	0.145
Shellfish:	
Oysters	0.029
Crabs	0.020
Lobsters	0.022
Vegetables:	
Spinach	0.024
Lentils	0.054
Beans	0.017
Mushrooms	0.018
Peas	0.018
Potatoes	0.002
String beans	0.002
Carrots	0
Lettuce	0.003
Cabbage	0.002
Asparagus	0.008
Cauliflower	0.008
Fruits	0
Bread	0
Eggs	0
Cereals	0
Butter	0
Milk	0
Cheese (except cream and dairy)	0
Cream cheese	0.005
Dairy cheese	0.022

Experiment. **Ingest a purine-free diet consisting of milk, egg, fruit, cheese, butter, sugar, and bread for one day. Continue the diet for breakfast and luncheon the next day but eat nothing after 12 o'clock noon, until 12 o'clock noon the following day, i.e., the third day of the experiment. At that time ingest 125–150 g. of gluten or some other purine-free protein preparation. On the fourth day of the experiment eat nothing until 9 P.M.**

Collect the urine each day in hour periods from 7 A.M. to 9 P.M. and analyze for uric acid (see methods on p. 905, ff.). Chart your data similarly to those shown in Figs. 261 and 262, and compare them with the findings there recorded.

7. The Rate of Purine Excretion. The purine material ingested by the average normal person and not transformed in the body is eliminated in about 24 hours. In the case of persons afflicted with gout the purine elimination is delayed. The establishment of this delayed purine elimination is often of diagnostic assistance.

Demonstrate the rate of purine excretion as follows: Ingest a purine-free diet consisting of egg, milk, cheese, starch, sugar, fruit, and water for two days and follow this by a day in which sweetbreads, thymus, or liver is substituted for one of the meals of the day (see table, p. 1053 for purine content of foods). Finish the experiment by ingesting the original purine-free diet for two days. Collect each day's urine and analyze for uric acid. How soon after the sweetbread ingestion was the original plane of endogenous uric acid elimination reestablished? If one desires to locate this time more definitely the urine may be collected in short periods (1 to 2 hours) and the uric acid content of each specimen determined. Particularly instructive data may be collected by performing the above experiment on a gout patient and upon a normal person for comparison.

8. A Study of Creatinine Elimination. It has been established that a normal person ingesting a creatinine-free diet will excrete a uniform quantity of creatinine from day to day. The daily excretion of an adult man of average weight ranges from 1–1.5 g. For data as to creatinine excretion of a 60-kg. man see Taylor and Rose's figures in the table on p. 1052. The creatinine excretion depends primarily on the active mass of protoplasmic tissue, and therefore, it is generally true that fat men will show a lower creatinine output than lean men of like body weight. The fact that, in a given individual, the creatinine output is uniform from day to day is made use of in metabolism experiments, for checking the completeness of 24-hour collections of urine. For further discussion of creatinine see p. 797.

Experiment. **Ingest an ordinary mixed diet (nonmeat) for a period of three days, varying the character of the diet daily. Collect the urine and analyze for creatinine. (See p. 899 for methods of analysis.)**

Did the creatinine elimination change with the change in diet?

9. Formation of Hippuric Acid in the Human Body. Hippuric acid is present in human urine in small amount, about 0.7 g. being excreted per day. The urine of herbivorous animals contains much larger quantities. This acid is formed in the liver in man, by the conjugation of benzoic acid and glycine; this formation is used clinically as a test of liver function. For procedure of the test, see Chapter 31.

10. The Partition of Urinary Nitrogen and Sulfur as Influenced by Diet. It was first shown by Folin[85] that the percentage of the total nitrogen and total sulfur of the urine which appeared in the form of any particular nitrogenous constituent or in any particular form of sulfur was regulated directly by the extent of the total nitrogen and sulfur elimination. This point is well illustrated in the table shown on p. 1055 which contains data regarding the so-called *partition* or *distribution* of the urinary nitrogen and sulfur.

It will be observed from an examination of this table that a normal protein diet which gave 16.8 g. of urinary nitrogen yielded 87.5 per cent of this nitrogen as urea, 3 per cent as ammonia, 3.6 per cent as creatinine, and 1.1 per cent as uric acid; whereas a nonprotein diet (starch and cream containing about 1 g. of nitrogen) which gave only 3.6 g. of urinary nitrogen yielded only 61.7 per cent of this nitrogen as urea but gave a greatly *increased percentage output* in the case of each of the other nitrogenous constituents mentioned, e.g., 11.3 per cent as ammonia, 17.2 per cent as creatinine, and 2.5 per cent as uric acid. The *percentage* output of neutral sulfur was also *greatly increased.*

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[85] Folin: *Am. J. Physiol.*, **13**, 118 (1905).

It will further be observed that the *actual daily output* of certain of the constituents is uninfluenced by the amount of protein ingested. Among these are creatinine and neutral sulfur. On the other hand, the output of inorganic sulfur and urea is more or less directly proportional to the protein ingestion. The observation of such facts as these led Folin to formulate his theory of protein metabolism, which held sway for many years, but which has recently been considerably modified (see discussion on p. 1021).

THE NITROGEN AND SULFUR PARTITIONS AS INFLUENCED BY DIET[85]

Constituent of the Urine	*Normal Protein Diet*			*Starch-cream Diet*		
	Amount, grams	*Nitrogen, grams*	*Per cent of total N or S*	*Amount, grams*	*Nitrogen, grams*	*Per cent of total N or S*
Urea	31.6	14.7	87.5	4.72	2.2	61.7
Ammonia	0.6	0.49	3.0	0.51	0.42	11.3
Creatinine	1.55	0.58	3.6	1.61	0.60	17.2
Uric acid	0.54	0.18	1.1	0.27	0.09	2.5
Undetermined N	..	0.85	4.9	..	0.27	7.5
Total N	..	16.8	100.0	..	3.6	100.0
Inorganic SO_3	3.27	..	90.0	0.46	..	60.5
Ethereal SO_3	0.19	..	5.2	0.10	..	13.2
Neutral SO_3	0.18	..	4.8	0.20	..	26.3
Total SO_3	3.64	..	100.0	0.76	..	100.0

Experiment. **During a period of two or three days ingest an ordinary mixed diet containing 100–125 g. of protein (16–20 g. of nitrogen) per day. Collect the urine accurately in 24-hour periods (p. 1044), preserve it, and analyze the urine of the second and third days for total nitrogen, urea, creatinine, total sulfur, inorganic sulfates, ethereal sulfates, and neutral sulfur (by difference). For methods of analysis see Chapter 31. Follow this period by a three-day period in which a diet of starch and cream having a similar calorific value is ingested. Analyze the urine for the second and third days as indicated above. Calculate your results and tabulate as shown in the table above. How did the change in the diet alter the metabolism of nitrogen and sulfur?**

In calculating the calorific value of a diet make use of the following values: protein or carbohydrate, 4 Calories per g.; fat, 9 Calories per g.

11. "Metabolic Product" Nitrogen in Feces. A certain quota of the fecal nitrogen is due to the presence of residues of digestive secretions, epithelial cells, bacteria, etc. The nitrogen in these forms has been called *metabolic product nitrogen.*

To determine this form of nitrogen one method[86] of procedure is as follows: Ingest a nonnitrogenous diet for a period of two days. The diet may include desired quantities of *starch, cream, sugar, butter, water, and sodium chloride.* About 15 g. *agar-agar* should be added to the diet to prevent constipation and to insure evacuation of approximately the normal quantity of feces. (For the influence of agar-agar, see Exp. 5, p. 1046.) To separate the feces properly, ingest a capsule of carmine at the beginning of the test and one of

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[86] For a discussion of other methods of estimating metabolic product nitrogen see Forbes, Mangels, and Morgan: *J. Agr. Research*, **9**, 405 (1917) and Schneider: *J. Biol. Chem.*, **109**, 249 (1935).

charcoal at the end (see p. 1046). Preserve the feces as described on p. 1046. After mixing the feces thoroughly, determine the nitrogen in weighed quantities by the Kjeldahl method,[87] according to directions given on p. 874. Calculate the quantity of nitrogen eliminated per day. Inasmuch as no nitrogen was ingested, the nitrogen present in the feces is of metabolic origin, i.e., it is made up principally of nitrogen in the form of cells, digestive secretions, and bacteria.

12. Influence of Defective Mastication on Food Residues in Feces. Rapid eating accompanied by defective mastication leads to the appearance of relatively large macroscopic food residues in the feces. Under some conditions, however, *protein utilization* (see p. 1047) may be as satisfactory after bolting of food as when it is very thoroughly masticated. This problem may be studied by the following method:

***a.* Ingest a diet containing meat and be certain to masticate the diet very thoroughly. Collect a stool, examine macroscopically; mix carefully and examine microscopically (see pp. 448 and 449).**

***b.* Ingest a diet similar to that employed in Exp. *a*, above. Bolt the food, i.e., ingest it practically without mastication. Examine the feces as above. Note the difference in the macroscopical and microscopical findings under (*a*) and (*b*).**

If the nitrogen of food and feces is determined, we may calculate the protein utilization (see Exp. 2, p. 1047). By the additional determination of urinary nitrogen, we may prepare a nitrogen balance (see Exp. 13).

13. Preparation of a Metabolic Balance. This test entails the analysis of the food ingested and of the urine and feces excreted, i.e., a study of the *income* and *outgo*. Proceed as follows:

Select a diet which is simple, i.e., consists of few constituents, and which lends itself readily to accurate chemical analysis. A good type of diet for ordinary metabolism experiments of this sort consists of crackers (graham or soda), milk, butter, water, and agar-agar (to prevent constipation). Meat, especially prepared in quantity sufficient for an entire experiment, may also be utilized. Ingest uniform quantities of these dietary constituents each day

BALANCE OF CALCIUM, MAGNESIUM, PHOSPHORUS, SULFUR, AND NITROGEN IN ACROMEGALY

	Calcium Oxide	*Magnesium Oxide*	*Phosphoric Anhydride*	*Sulfur*	*Nitrogen*
	Grams				
Ingestion (daily)	1.494	0.486	3.192	1.190	18.84
Excretion (urine)	0.159	0.160	1.701	1.006	17.60
Excretion (feces)	1.093	0.226	1.002	0.135	1.10
Excretion (total)	1.252	0.386	2.703	1.141	18.70
Retention (daily)	0.242	0.100	0.489	0.049	0.14
Retention (per cent)	16.2	20.6	15.3	4.1	0.7

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[87] In the oxidation process use 10 g. of potassium sulfate instead of the copper sulfate. The remainder of the procedure is the same as for urine.

for a period of three days. Make an accurate collection of the urine passed during this interval (see p. 1044). Separate the feces representing the three-day period (see p. 1046), and analyze foods, urine, and feces. The balances ordinarily prepared are those for nitrogen, sulfur, phosphorus, and calcium. Analytical methods for the determination of these elements may be found in Chapter 31.

The table on p. 1056 includes balances obtained in a metabolism test in a case of acromegaly.

14. The Influence of Water on Metabolism. It has been demonstrated that increased water ingestion influences favorably many of the functions and activities of the human body.[88] The increase in protein catabolism which accompanies high water intake is shown in the following data collected from an experiment upon a normal man. In this experiment the water ingestion *at meals* was increased 3 liters per day during the water period.

INFLUENCE OF HIGH WATER INTAKE UPON URINE VOLUME AND NITROGEN PARTITION

Day of Period	*Urine Volume, ml.*	*Nitrogen, g.*	*Urea Nitrogen, g.*	*Ammonia Nitrogen, g.*	*Creatinine Nitrogen, g.*	*Creatine Nitrogen, g.*
Preliminary Period						
4	830	12.987	11.338	0.288	0.629	
5	920	12.084	11.476	0.305	0.619	
6	880	13.183	11.568	0.369	0.651	
Water Period						
1	3440	14.161	12.596	0.486	0.610	0.063
2	3840	13.491	11.583	0.499	0.616	0.024
3	3670	12.981	11.212	0.553	0.589	0.102
4	3610	12.976	11.455	0.485	0.608	0.055
5	4020	13.138	11.879	0.456	0.589	0.128

The above data indicate an increased catabolism of protein material as shown by an increased output of total nitrogen upon the first and second days of the water period. Part of this increase may, however, have been due to a flushing of the tissues rather than to increased catabolism of protein structures.

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[88] Hawk: "The relationship of water to certain life processes and more especially to nutrition." Read before *American Philosophical Society*, Philadelphia, Feb., 1914. (See *Biochem. Bull.*, 3, 420 (1914); also "Water as a Dietary Constituent," in *Endocrinology and Metabolism*, New York and London, D. Appleton and Co., 1924, Vol. 3, p. 277.) The so-called "water intoxication" demonstrated by Rowntree (*Arch. Int. Med.*, **32**, 157 (1923)) and later by Underhill and Sallick (*J. Biol. Chem.*, **63**, 61 (1925)) cannot be advanced as an argument against the value of a high water intake for the average individual. The observers mentioned experimented on animals and introduced as much as 50 ml. water per kg. body weight every half hour until a definite toxic result was obtained. A similar excessive ingestion of any standard food would probably produce results fully as serious.

a. RELATION OF WATER INTAKE TO VOLUME AND SPECIFIC GRAVITY OF THE URINE. Ingest an ordinary mixed diet for two days. Collect the urine in 24-hour periods. During the first day ingest very little fluid of any kind either at meals or between meals. On the second day ingest as much water as you can without physical inconvenience. A person of average size should have no difficulty in drinking 5–6 liters per day.

Measure the volume of each day's urine and take the specific gravity. Note the pronounced increase in volume and the low specific gravity of the urine under the influence of high water ingestion.

b. INFLUENCE ON PROTEIN CATABOLISM. That water stimulates protein catabolism may easily be demonstrated as follows: Ingest a uniform diet (milk, crackers, butter, peanut butter, and water) for a period of four days. During the first two days ingest your customary volume of water per day. During the last two days increase the water ingestion to 5–6 liters per day. Collect urine in 24-hour periods and analyze for total nitrogen by Kjeldahl method (see p. 874). Note the increased excretion of nitrogen under the influence of high water intake. If time permits, other nitrogenous urinary constituents may be determined (see table above).

c. INFLUENCE OF WATER DEFICIENCY. The importance of water in nutrition may be shown very satisfactorily in guinea pigs. Proceed as follows: Place two young pigs (150–200 g.) in separate cages, and give each free access to a diet of hay, oats, and lemon or orange juice which has been dried rapidly at a low temperature. Permit one pig water ad lib., and give the second pig no water. The pig receiving water will remain normal and will exhibit normal gain in body weight. The pig receiving no water will soon show pronounced losses in body weight and other signs of abnormality. The animal will die in a short time unless water is added to the diet. This experiment demonstrates very clearly that water is an indispensable dietary constituent. In fact, water is more important than food. The following experiment will show this.

d. FOOD STARVATION VS. WATER STARVATION. Place two young guinea pigs (150–200 g.) in separate cages. Give one a diet such as that described above, plus orange juice (5 ml.) but no water. Give the second pig no food, but permit free access to water. The pig receiving no water will quickly become abnormal, and it will be necessary to give it water to preserve its life. The second pig, which has access to water[89] but receives no food to eat, will live longer than the pig receiving an abundance of dry food. This little experiment impresses the important fact that man can live longer without food than without water. By restricting the amount of water in the diet of an albino rat, the animal may be kept at constant body weight for several weeks although the diet is otherwise adequate. In many respects the effect is similar to that of underfeeding. Some increased tolerance to water restriction may develop during such a test.

15. ***The Metabolism of Fasting.***[90] The metabolism of a fasting man is entirely different from the metabolism of a well-nourished person. The collection and analysis of the urine during a short fast (three to seven days) will demonstrate many important facts. The following table, which contains data from fasting tests made in the senior author's laboratory,[91] illustrates some of the points in which fasting metabolism differs from normal metabolism:

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[89] In case the pig does not drink the water, the animal should be fed the fluid by a sound.

[90] For discussions of fasting see: Benedict: *A Study of Prolonged Fasting*, Carnegie Inst. Wash. Pub., 203, 1915; Morgulis: *Fasting and Undernutrition*, New York, E. P. Dutton and Co., 1923; and Jackson: *Inanition and Malnutrition*, Philadelphia, P. Blakiston's Son and Co., Inc., 1925.

METABOLISM IN FASTING

Day of Period	*Body Weight, kg.*	*Total N, g.*	*Ammonia N, g.*	*Creatine N, g.*	*Acidity ml. 0.1 N NaOH*	P_2O_5 *g.*	*Chloride as NaCl, g.*
Preliminary Feeding Period							
1–4	Av. 74.16	10.430	0.112	None	238.6	2.768	9.007
Fasting Period							
1	73.32	10.072	0.288	0.269	328.9	2.616	5.035
2	71.98	15.072	0.642	0.073	677.1	2.509	3.231
3	70.92	14.463	0.862	0.089	770.4	2.851	2.539
4	70.24	13.080	1.201	0.068	664.2	2.490	1.253
5	69.61	11.801	1.266	0.033	525.0	2.376	1.474
6	69.12	11.214	1.373	0.022	462.4	1.186	1.132
7	68.70	10.734	1.371	0.003	438.9	0.955	1.137

Abstinence from food for a few days can in no way operate to the disadvantage of a normal person. In fact, individuals affected with certain types of gastrointestinal disorders are benefited by fasting. The *fasting treatment* has also been used in cases of diabetes mellitus and in the treatment of obesity.

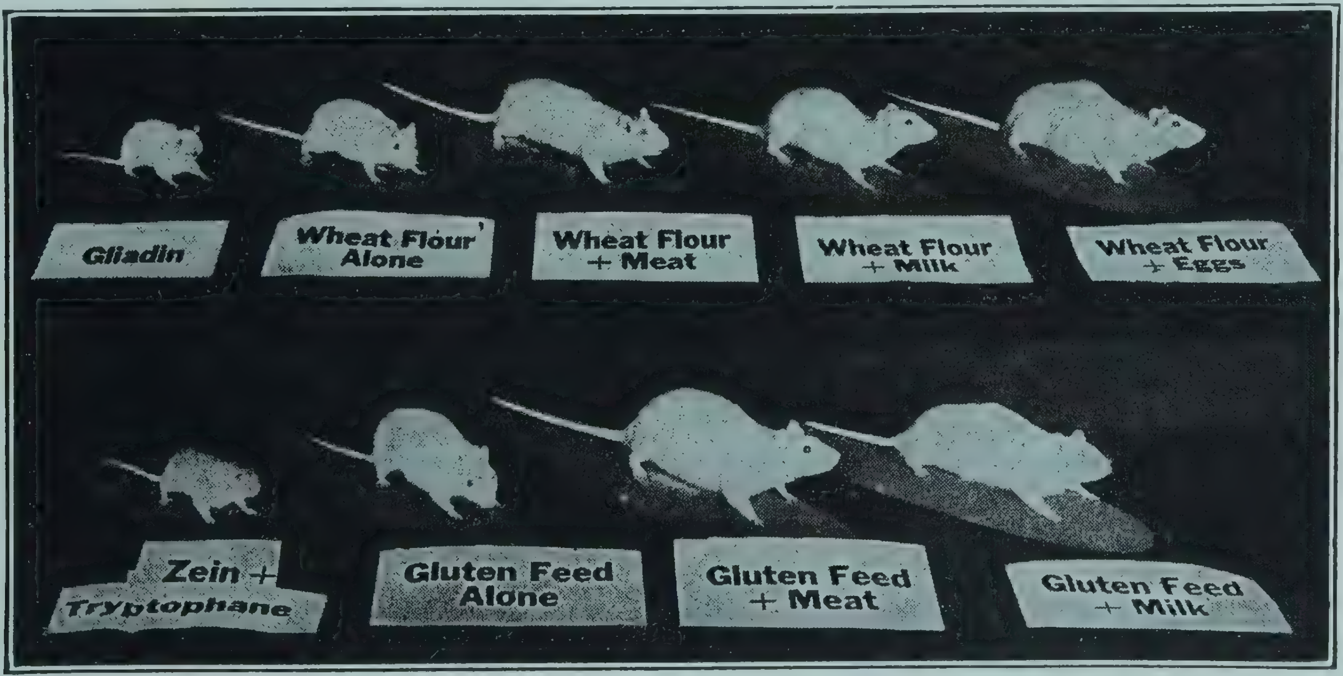

FIG. 263. SHOWING IMPORTANCE OF ADEQUATE PROTEIN (AMINO ACIDS) IN THE DIET.

Courtesy, Mendel: *Nutrition—The Chemistry of Life*, New Haven, Yale University Press, 1923.

In order to determine experimentally how the fasting metabolism differs from normal metabolism, proceed as follows: Ingest an ordinary mixed diet and collect your urine (see p. 1044) for a day. Measure the volume and analyze the sample for total nitrogen, ammonia, creatine, sodium chloride, total

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[91] The chloride, phosphate, and acidity determinations were collected during one seven-day fast and the other data collected during a different fast on the same man.

phosphates, and acidity[92] (for methods see Chapter 31). For the next few days (three to seven as desired) ingest nothing but water and collect the urine accurately and analyze for the constituents enumerated above. Tabulate your results and compare them with those given in the table above.

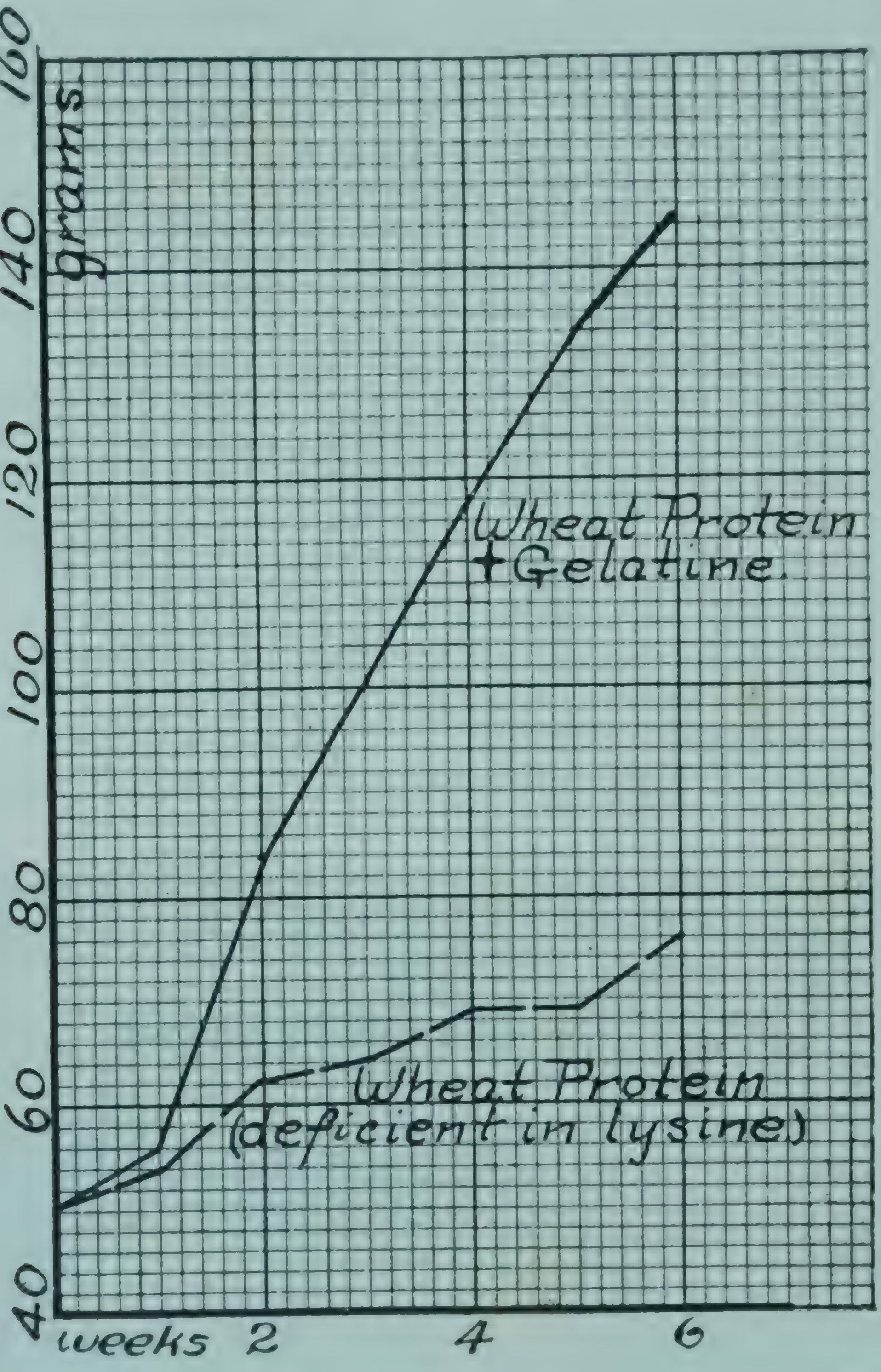

FIG. 264. CURVE SHOWING INFLUENCE OF A DEFICIENCY OF LYSINE IN THE DIET.

Unpublished data from the senior author's laboratory.

16. Influence of Protein (Amino Acid) Deficiency. Certain of the amino acids which occur in proteins cannot be synthesized in the animal body. This subject is discussed on p. 1015. The importance of proper protein (amino acids) in the diet is illustrated in Fig. 263. The following experiment, which may readily be made using white rats as subjects, will clearly demonstrate the importance of the amino acid lysine.

Demonstration of Lysine Deficiency. **Place two young white rats (40–60 g.) in separate cages. Feed one rat Diet 1 and the other Diet 2 as listed in the following table.**

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[92] A more accurate experiment may be carried out by ingesting a uniform diet of known composition (see p. 1047) for a few days before the fast.

LYSINE DEFICIENCY DIET

	Diet 1 *per cent*	*Diet 2* *per cent*
Rolled oats[93]	60	60
Gelatin[93]	0	10
Dextrin or starch	30	20
Salt mixture	4	4
Hydrogenated vegetable oil	5	5
Cod liver oil	1	1

The rat receiving Diet 2 will grow normally because of the high lysine content of gelatin. The animal receiving Diet 1 will fail to grow properly because of lysine deficiency. See Fig. 264.

III. MICROBIOLOGICAL DETERMINATION OF AMINO ACIDS

The widespread use of microorganisms for the assay of the vitamins in the B-complex group soon revealed their requirements for the amino acids. Extensive investigations followed and successful quantitative assays for 14 amino acids, using bacteria, appeared shortly. At least three amino acids may be determined with mutant strains of the mold *Neurospora* (see p. 1066), and one, L-lysine, with a specific decarboxylase. Of the amino acids recognized as physiological constituents, only norvaline, norleucine, and hydroxyproline have not been found essential for any organism yet investigated. The fundamental principle involved in microbiological assays is to measure the response of bacteria, yeasts, or molds to graded increments of the sample and of a standard solution added to media furnishing all the nutrients required by the microorganism except the amino acid (or other nutrient) under assay. The graded response may be measured by the increase in population of the microorganisms (i.e., turbidimetrically) or by their products of metabolism (acid or CO_2 production).

Microbiological methods are advantageous in that several amino acids may be determined in a single prepared hydrolyzate with the same microorganism with only slight modification of the basal medium. Only the natural or L-forms are biologically active, except in the case of aspartic acid, whose D- and L-isomers are equally available to *Lactobacillus delbrückii*. However, the L-forms are more expensive than the synthetic DL-mixtures, so that the latter are used in the basal medium and in the standard series when available. When substituting one form for the other, or when the hydrochlorides are used, appropriate adjustments should be made in the amounts employed. Microbiological methods are exceedingly simple compared to the chemical isolation procedures. Moreover, they are not subject to isolation losses since determinations are made directly on the hydrolyzates.

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[93] Oat protein is low in lysine. Gelatin is relatively high in this amino acid (see p. 122).

Careful attention must be given to proper methods of preparing hydrolyzates for assay. Acid is known to destroy tryptophan and, in the presence of carbohydrate, tyrosine. Enzymatic digestion or hydrolysis with barium hydroxide should be employed for tryptophan assays, since hydrolysis with sodium hydroxide has been reported to give erratic results. Alkaline hydrolysis racemizes the amino acids, so that such treatment must be continued until the racemization is complete, and the values found multiplied by two. Unless there is complete assurance that no amino acid loss occurs during the preliminary preparation of hydrolyzates, caution should be observed in applying such analytical data to the composition of the intact protein.

AMINO ACID REQUIREMENTS OF VARIOUS LACTIC ACID BACTERIA*

Amino Acid†	*Lactobacillus arabinosus*	*Lactobacillus casei*	*Lactobacillus delbrückii*	*Streptococcus faecalis*	*Streptococcus lactis*	*Leuconostoc mesenteroides*
Arginine	±	+	+	+	+	+
Histidine	−	±	−	−	±	+
Isoleucine	+	±	+	+	+	+
Leucine	+	+	+	+	+	+
Lysine	±	±	±	+	±	+
Methionine	±	±	±	−	+	+
Phenylalanine	±	+	+	−	±	+
Threonine	±	±	±	+	−	+
Tryptophan	+	+	+	+	−	+
Valine	+	+	+	−	+	+
Alanine	−	±	±	+	−	±
Aspartic acid	±	+	+	+	−‡	+
Cystine	+	+	+	±	−	+
Glutamic acid	+	+	+	+	−‡	+
Glycine	−	−	−	+	−	+
Proline	−	−	−	−	−	+
Serine	−	+	+	+	±	+
Tyrosine	±	+	+	±	−	+

* Snell: *Conference on Amino Acid Analysis of Proteins*, New York Academy of Sciences, 1945.

† The symbol + indicates that the amino acid is essential; ±, that some growth but not maximum growth occurs in its absence; and −, that the amino acid is not essential.

‡ Asparagine and glutamine were essential for growth, and supplied the organism with these amino acids.

The qualitative amino acid requirements of six lactic acid bacteria are shown in the table above. These microorganisms have been employed in the quantitative determination of 14 amino acids. To facilitate the application of these methods, a typical procedure is described below, and pertinent data regarding the preferred methods of preparation of the hydrolyzates, the concentrations of the amino acids at half-maximum and at maximum growth, and the nature of the responses measured are

MICROBIOLOGICAL DETERMINATION OF THE AMINO ACIDS

Amino Acid	*Hydrolytic Agent**	*Test Microorganisms†*	*Concentrations of L-isomer*		*Response Measured*	*References for Preparation of Media and Details of Tests*
			At Half Maximum Growth	*At Maximum Growth*		
			Micrograms per 10-ml. tube			
Arginine	10 per cent HCl	*Streptococcus faecalis*	25	100	Acidity	*J. Biol. Chem.*, **160,** 35 (1945)
		Lactobacillus casei	15	50	Turbidity	" " " **152,** 83 (1944)
Histidine	10 per cent HCl	*Streptococcus faecalis*	13	50	Acidity	*J. Biol. Chem.*, **160,** 35 (1945)
		Leuconostoc mesenteroides P-60	20	50	Acidity	" " " **159,** 653 (1945)
Isoleucine	10 per cent HCl	*Lactobacillus arabinosus 17-5*	13	50	Acidity	*J. Biol. Chem.*, **150,** 305 (1943); **152,** 83 (1944)
		Streptococcus faecalis	40	100	Acidity	" " " **160,** 35 (1945)
Leucine	10 per cent HCl	*Lactobacillus arabinosus 17-5*	18	60	Acidity	*J. Biol. Chem.*, **150,** 305 (1943); **152,** 83 (1944)
		Streptococcus faecalis	30	100	Acidity	" " " **160,** 35 (1945)
Lysine	10 per cent HCl	*Leuconostoc mesenteroides P-60*	75	175	Acidity	*J. Biol. Chem.*, **156,** 715 (1944)
		Streptococcus faecalis	75	200	Acidity	" " " **160,** 35 (1945)
Methionine	10 per cent HCl	*Streptococcus faecalis*	15	75	Acidity	*J. Biol. Chem.*, **160,** 35 (1945)
Phenylalanine	10 per cent HCl	*Lactobacillus delbrückii LD5*	20	60	Acidity	*J. Biol. Chem.*, **160,** 35 (1945)
		Leuconostoc mesenteroides P-60	..	50	Acidity	" " " **161,** 643 (1945)
		Lactobacillus casei	15	60	Acidity	" " " **151,** 511 (1943)
Threonine	10 per cent HCl	*Streptococcus faecalis*	30	100	Acidity	*J. Biol. Chem.*, **160,** 35 (1945)
Tryptophan‡	5 N NaOH or 5 N $Ba(OH)_2$ (or enzyme digestion)	*Streptococcus faecalis*	4	15	Acidity	*J. Biol. Chem.*, **160,** 35 (1945)
		Lactobacillus arabinosus 17-5	4	15	Acidity	" " " **155,** 1 (1944)
Valine	10 per cent HCl	*Lactobacillus arabinosus 17-5*	15	40	Acidity	*J. Biol. Chem.*, **150,** 305 (1943); **152,** 83 (1944)
		Streptococcus faecalis	30	100	Acidity	" " " **160,** 35 (1945)
Aspartic acid	10 per cent HCl	*Lactobacillus delbrückii LD5*	300	800	Acidity	*J. Biol. Chem.*, **157,** 651 (1945)
Glutamic acid	10 per cent HCl	*Lactobacillus arabinosus 17-5*	120	400	Acidity Turbidity	*J. Biol. Chem.*, **159,** 273 (1945); **152,** 83 (1944)
Serine	10 per cent HCl	*Lactobacillus delbrückii LD5*	100	250	Acidity	*J. Biol. Chem.*, **157,** 651 (1945)
Tyrosine	5 N NaOH§	*Lactobacillus delbrückii LD5*	25	75	Acidity	*J. Biol. Chem.*, **163,** 159 (1946)

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* Samples are hydrolyzed ten hours at 120° C.

† These microorganisms may be obtained from the American Type Culture Collection, Washington 6, D. C., under the following numbers: *Streptococcus faecalis*, No. 9790; *Lactobacillus casei*, No. 7469; *Leuconostoc mesenteroides* P-60, No. 8042; *Lactobacillus arabinosus* 17-5, No. 8014; *Lactobacillus delbrücki* LD5, No. 9595. Prepared culture media for microbiological assay of the amino acids may be obtained from H. M. Chemical Co., Ltd., Santa Monica, California; Difco Laboratories, Inc., Detroit 1, Mich.

‡ Alkali racemizes tryptophan. Though hydrolysis with sodium hydroxide has been found satisfactory by some investigators, others [Woolley and Sebrell: *J. Biol. Chem.*, **157,** 141 (1945)] have obtained erratic results. The latter prefer enzymatic digestion which avoids possible variable racemization or destruction of tryptophan, or hydrolysis with barium hydroxide. Indole and anthranilic acid can replace tryptophan for *L. arabinosus* and should be removed from hydrolyzates by ether extraction at pH 4.0.

§ Alkaline hydrolysis racemizes tyrosine. Thus, values should be multiplied by 2. Acid hydrolysis of samples containing appreciable amounts of carbohydrate should be avoided because the resultant humin formation is accompanied by a loss of tyrosine.

summarized in the table on p. 1063. References are made to the original publications for further details.[93a]

1. Microbiological Determination of Isoleucine, Leucine, and Valine (Method of Shankman):[94] Principle. Isoleucine, leucine, and valine are determined by measurement of the growth stimulation of *Lactobacillus arabinosus*. Samples are prepared for assay by the microhydrolytic procedure of McMahan and Snell,[95] employing 10 per cent hydrochloric acid. Each amino acid is determined separately by omitting it from the basal medium.

Preparation of the Sample. Weigh into a pyrex test tube an aliquot of the sample containing 100 mg. of protein. Add 1 ml. of 10 per cent hydrochloric acid[96] and seal the tube in an oxygen flame. Heat for 10 hours in an autoclave at 15 lb. pressure, or in an oven at 120° C. Cool, open the tube, and wash out with approximately 95 ml. of water. Adjust with 4 N sodium hydroxide to pH 6.8 to 7.0 and dilute to a concentration[97] of approximately 7.5 μg. of L-valine, 15 μg. of L-leucine, or 10 μg. of L-isoleucine per ml.

Preparation of Basal Medium. The composition of the basal medium is given in the following table:

COMPOSITION OF BASAL MEDIUM FOR THE ASSAY OF LEUCINE, ISOLEUCINE, AND VALINE

(The amounts shown are for the preparation of 500 ml. of basal medium)

Glucose	5 g.	L(−)-Leucine	100 mg.
Sodium acetate	3 g.	DL-Isoleucine	100 mg.
Salt solution A	5 ml.	DL-Valine	100 mg.
Salt solution B	5 ml.	L(−)-Cystine	50 mg.
Adenine	5 mg.	DL-Methionine	50 mg.
Guanine	5 mg.	L(−)-Tryptophan	17 mg.
Uracil	5 mg.	L(−)-Tyrosine	17 mg.
Thiamine	50 μg.	DL-Phenylalanine	50 mg.
Riboflavin	100 μg.	L(+)-Glutamic acid	200 mg.
Nicotinic acid	100 μg.	DL-Threonine	100 mg.
Biotin	0.2 μg.	DL-Alanine	100 mg.
Pyridoxine	50 μg.	L(−)-Asparagine or aspartic acid	200 mg.
Pantothenic acid	50 μg.	L(+)-Lysine	100 mg.
p-Aminobenzoic acid	5 μg.	L(+)-Arginine	25 mg.

For use in the assay tubes, omit from the basal medium that amino acid for which analysis is being made. Adjust to pH 6.6 to 6.8 with 4 N NaOH after

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[93a] For discussion of additional microbiological assays of amino acids the reader is referred to reviews by Snell: *Adv. in Protein Chem.*, **2**, 85 (1945); Schweigert and Snell: *Nutrition Abst. and Revs.*, **16**, 497 (1947); Dunn: *Food Technology*, **1**, 269 (1947); Barton-Wright: *The Microbiological Assay of the Vitamin B-Complex and Amino Acids*, New York, Pitman Publishing Corp., 1952.

[94] Shankman: *J. Biol. Chem.*, **150**, 305 (1943).

[95] McMahan and Snell: *J. Biol. Chem.*, **152**, 83 (1944).

[96] Reagent grade concentrated hydrochloric acid solution contains approximately 35 per cent hydrochloric acid.

[97] Estimate the approximate concentrations of the amino acids present from the composition of the test material.

preparation. Store in the refrigerator and arrange storage flask with a siphon so that the amount required for the day's work may be readily obtained.

The amino acids may be weighed out directly or may be prepared as stock solutions in water, containing the desired amount in a few ml. Where necessary, a little hydrochloric acid may be used to aid in solution. Store the stock solutions in the refrigerator.

Salt solution *A* contains 25 g. each potassium monohydrogen phosphate and potassium dihydrogen phosphate, in 250 ml. water. Salt solution *B* has the following composition: 10 g. magnesium sulfate heptahydrate, 0.5 g. sodium chloride, 0.5 g. ferrous sulfate heptahydrate, and 0.5 g. manganese sulfate tetrahydrate, in 250 ml. water. Add a few drops of hydrochloric acid to solution *B* to keep from precipitating.

Prepare stock solutions of adenine, guanine, and uracil, containing 1 mg. per ml. Solution is aided by heating in the presence of a few drops of hydrochloric acid. Store in the refrigerator, and renew at frequent intervals.

Prepare stock solutions in water of thiamine, calcium pantothenate, and pyridoxine, containing 100 μg. per ml. Store in the refrigerator and renew at frequent intervals. Prepare the riboflavin stock solution in 0.02 N acetic acid, to contain 100 μg. per ml. Store as above, and in addition keep from exposure to light.

Stock solutions of nicotinic acid (100 μg. per ml.) and biotin (2 μg. per ml.) are prepared in 50 per cent ethyl alcohol. The stock solution of *p*-aminobenzoic acid (50 μg. per ml.) is prepared in glacial acetic acid, and stored in a dark-glass bottle.

Procedure. **Carry stab cultures[98] of *Lactobacillus arabinosus 17-5* on yeast extract–dextrose–agar (Difco) and subculture monthly. After transfer, incubate the cultures at 30° C. for 24 to 48 hours, and then hold in the refrigerator. Prepare the inoculum for the assay tubes by transfer from the stock culture to a sterile centrifuge tube of the *complete* basal medium, containing all the amino acids listed. Incubate the inoculum at 30° C. for 24 hours before use. Centrifuge aseptically and wash 3 times by suspending in sterile 0.9 per cent saline and centrifuging. Finally suspend the cells in 30 ml. of saline.**

Assay. **Pipet into pyrex test tubes 0, 0.25, 0.50, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 ml. aliquots of the test extract. Add to each tube 5 ml. of basal medium lacking the particular amino acid to be determined, and sufficient distilled water to make a total volume of 10 ml. Prepare a similar series of standards containing the amino acid to be assayed, employing a pure solution of the appropriate amino acid in place of the test extract. Optimal concentrations are 15 μg. DL-valine or L-leucine per ml. or 20 μg. DL-isoleucine per ml. Plug the tubes with cotton and sterilize in an autoclave at 15 pounds pressure for 15 minutes. After cooling to room temperature, add one drop (0.03 ml.) of inoculum to each tube and incubate at 30° C. for 72 hours.**

Transfer the contents of each tube to a 125-ml. Erlenmeyer flask, using a constant volume of distilled water as a wash. Titrate the lactic acid produced with 0.05 N sodium hydroxide using bromothymol blue as the indicator.

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[98] American Type Culture Collection, No. 8014.

CALCULATION. For each amino acid assayed, plot the titrations for the standard series in ml. of 0.1 N sodium hydroxide against μg. of the standard used. From the appropriate curve, estimate the amount of each amino acid assayed per tube. Calculate the concentration per ml. of test extract. Determine the amino acid content of the test sample from the values obtained from not less than five of the tubes which do not vary by more than ±15 per cent from the average. Synthetic DL-mixtures contain 50 per cent of the biologically active enantiomorph. If the racemic mixture was used as the standard, multiply the value obtained by one-half.

In Fig. 265 is shown a standard curve obtained with DL-valine, during the assay of a β-lactoglobulin preparation for that amino acid. The method of calculation is illustrated in the table on p. 1067. Results are expressed as per cent of valine in the protein constituent of the material assayed.

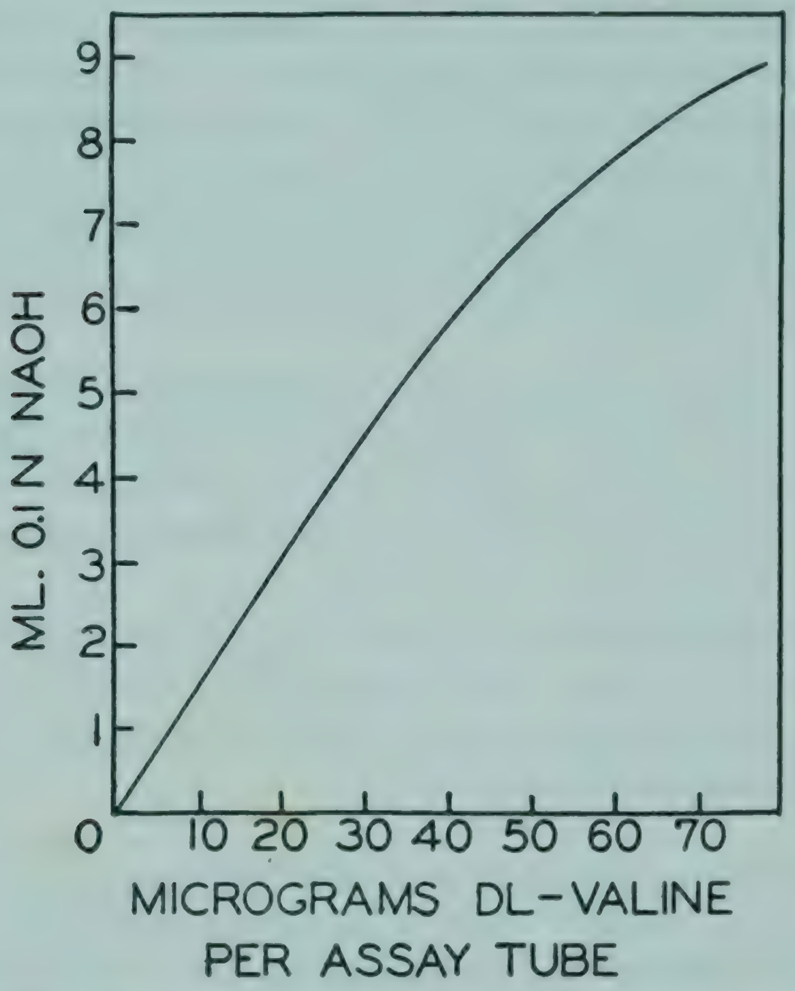

FIG. 265. TITRATION OF LACTIC ACID PRODUCED BY *Lactobacillus arabinosus* GROWN AT DIFFERENT LEVELS OF VALINE SUPPLEMENTATION.

There is some evidence that in addition to a proper balance of the essential amino acids, it may be necessary to supply certain peptide linkages in the dietary to insure satisfactory utilization of protein nitrogen. Thus, it has been demonstrated in rat assays that omission of a new factor, strepogenin, from a dietary including a hydrolyzate containing all of the essential amino acids in satisfactory quantities was responsible for a poor biological response. This factor, believed to be a peptide, is a growth stimulant for *Lactobacillus casei* and *Streptococcus lactis*. A preliminary method of assay has appeared based upon the growth of the former microorganism.[99]

2. Use of *Neurospora* Mutants. In 1941 Beadle and Tatum[100] reported the production of biochemical mutants in the red bread mold *Neurospora* by irradiation with ultraviolet and x-rays. These mutants are unable to carry out certain specific chemical reactions which are normally possible to the unmutated or wild-type strain. For example, the untreated mold is able to synthesize all the components of protoplasm it needs (vitamins, amino acids, etc.) on a medium containing only sucrose, nitrate, inorganic salts, and biotin. Various mutant strains have been obtained which are unable to grow without the addition of certain specific vitamins or amino acids to the medium, i.e., the strain has lost the ability it at one time possessed to synthesize that particular vitamin or amino acid. It is believed that this loss of specific biochemical power is due to the loss of a single gene, which would ordinarily control the particular reaction which no longer occurs in the mutant.

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[99] Sprince and Woolley: *J. Exp. Med.*, **80**, 213 (1944).

[100] Beadle and Tatum: *Proc. Nat. Acad. Sci.*, **27**, 499 (1941); Tatum and Beadle: *ibid.*, **28**, 234 (1942).

MICROBIOLOGICAL ASSAY FOR VALINE IN A β-LACTOGLOBULIN PREPARATION
(N = 14.6 PER CENT)*
(0.107 g. was hydrolyzed and diluted at 750 ml.)

Test Extract Added to Assay Tube	*Titration after Incubation*	DL-*Valine Equivalent Evaluated from Standard Curves*	DL-*Valine per ml. of Test Extract*	
ml.	*ml. 0.1 N NaOH*	μg.	μg.	
0	0	0	. .	
0.25	0.7	5.1	20.4 (omit)	
0.50	1.2	8.1	16.2	Average 15.3
0.75	1.8	12.2	16.3	
1.0	2.2	14.7	14.7	
1.5	3.3	22.0	14.7	
2.0	4.5	30.3	15.2	
3.0	6.5	46.5	15.5	
4.0	7.9	62.0	15.5	
5.0	8.6	72.7	14.5	

Calculation:

15.3 × ½ × 750 = 5.74 mg. of L-valine per 100 mg. protein in sample.

* The theoretical nitrogen content of β-lactoglobulin is 15.6 per cent. 0.107 g. contains 100 mg. of protein.

The implications of these remarkable findings are obviously widespread, not only in the field of genetics but also in many other ways, one of which is in the field of microbiological assay. The *Neurospora* mutants with specific nutritional defects can be used for microbiological assay of amino acids and other nutrients just as has been described for the use of certain bacteria. For example, Horowitz and Beadle[101] have described a *cholineless* strain of *Neurospora*, i.e., a mutant which cannot synthesize choline and is therefore unable to grow without the presence of added choline in the medium. These authors have described a method for the microbiological assay of choline by the use of this strain. The procedure is substantially identical in principle with that described on p. 1064 for the microbiological assay of amino acids, except that growth response is followed by drying and weighing the mold mycelium. Comparison is made in terms of a standard curve obtained with known amounts of choline. Methods have also been described for the assay of certain amino acids (methionine, leucine, arginine) by the use of the proper *Neurospora* mutants, and in general this versatile phase of microbiological assay appears to be just beginning in so far as future application is concerned.

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[101] Horowitz and Beadle: *J. Biol. Chem.*, **150**, 325 (1943). See Chapter 35, "Choline."

Another contribution of this work is to facilitate knowledge concerning gene action, and the reactions controlled by genes. If, as has been suggested, each step in a biochemical reaction is under the control of a single gene, studies of the various mutants should permit a better understanding of the intermediate stages in biochemical reactions. For example, by this method of approach the progressive reaction ornithine → citrulline → arginine, postulated by Krebs and Henseleit in connection with urea synthesis in mammalian liver (see p. 1039) has been shown to occur in distinct gene-controlled steps in *Neurospora*. While this does not necessarily mean that the reaction occurs in this way, or even occurs at all, in higher cells, it at least indicates the possibility of it so doing. There is no doubt that further studies of this type will add significantly to our knowledge of the fundamental chemical processes of protoplasm.

IV. EXPERIMENTS ON CARBOHYDRATE METABOLISM

1. Hyperglycemia Produced by Carbohydrate Ingestion. The average glucose content of normal blood is somewhat less than 0.1 per cent. This is increased (hyperglycemia) after the ingestion of carbohydrate food. The increase is noted more quickly after the ingestion of monosaccharides than after the ingestion of the more complex carbohydrates. After the ingestion of 100 g. of glucose the increase in the sugar of the blood sometimes occurs in three minutes.

(a) Influence of Glucose. **In the morning before breakfast, or at least three hours after breakfast, determine the normal sugar content of your blood by means of some accurate micromethod (see Chapter 23). Ingest 100 g. of glucose dissolved in 250 ml. of water, and again determine the blood sugar level at intervals of 5, 15, and 30 minutes, and one, two, and three hours. (Plot a curve similar to the one shown in Fig. 266.) The urine may also be examined for sugar at intervals of one hour after the sugar ingestion.**

Repeat the experiment on another day using 250 g. of glucose and compare the results with those obtained after the ingestion of 100 g. Explain your findings. If desired, this experiment may be combined with the one on "Alimentary Glucosuria," below.

(b) Influence of Starch. **Repeat the experiment as given above for glucose except that 170 g. of white bread or 100 g. of starch made into a paste[102] are substituted for the 100 g. of glucose.**

The experiment may be repeated as described above, using an increased amount of starch.

The various experiments may be conducted on patients suffering from diabetes mellitus if such are available and instructive data collected. The alimentary hyperglycemia will generally be slower in reaching its maximum and will be more prolonged than in the case of normal subjects. In some instances after the diabetic has ingested

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[102] In making starch paste, rub up the dry starch in a mortar with cold water, pour the suspended starch granules into boiling water, and stir.

100 g. of glucose the blood sugar does not reach its maximum until a period of two hours has elapsed. The blood sugar also returns to its former level more slowly than in the case of normal individuals.

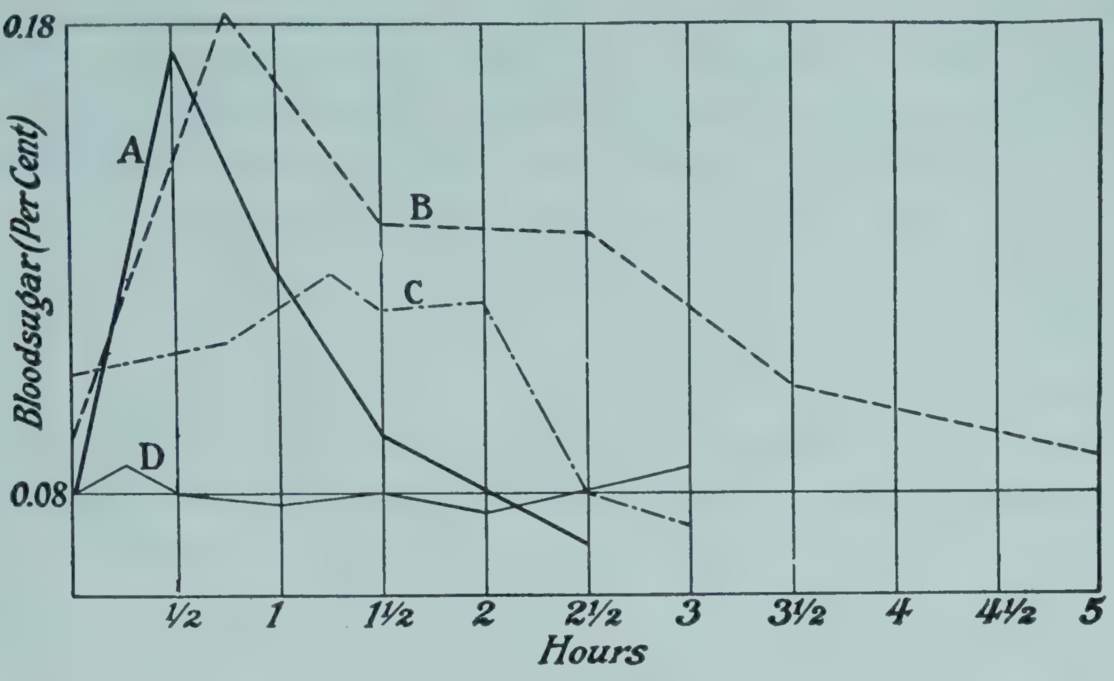

FIG. 266. BLOOD SUGAR AS INFLUENCED BY DIET.
A, glucose; *B*, starch; *C*, starch and fat; *D*, fat.

2. ***Experiments on Alimentary Glucosuria.*** According to Folin and Berglund, the sugar of normal urine consists of a motley variety of carbohydrate products and carbohydrate derivatives including di- and polysaccharides. These are believed to arise from foreign, unusable carbohydrate materials present in grains, vegetables, and fruits and from decomposition products due to cooking, canning, and baking of food. The ingestion of pure glucose, fructose, maltose, dextrin, or starch does not normally give rise to glucuresis, but the ingestion of impure products or of an ordinary carbohydrate meal increases the sugar of the urine. So also may lactose and galactose.

Procedure. **On arising at 7:00 A.M., the student should empty his bladder and discard the urine voided. He should then drink one glass of water but eat no food. At 8:00 A.M., the bladder is again emptied and the urine kept for analysis. The student then immediately drinks 200 g. of pure glucose dissolved in about 500 ml. of water. Urine specimens are collected again at 9:00, 10:00, and 11:00 A.M. Test each specimen for sugar by Benedict's qualitative reduction test. Determine sugar in each specimen according to the method of Folin and Svedberg (see Chapter 31).**

Other students should go through the same procedure with the exception that for glucose there should be substituted 200 g. of cane sugar, 200 g. of commercial dextrin, 200 g. of pure dextrin-starch,[103] 100 g. of pure lactose, a dozen graham crackers with water only, two baked apples, bread and butter, meat with water only, or a large dish of pure gelatin without added sugar.

This experiment may be made more complete by making determinations of blood sugar at short intervals as described in Exp. 1, p. 1068. If desired, data on glucosuria, hyperglycemia, and carbohydrate in feces (see below) may be collected from one experiment.

3. ***Effect of Exercise on the Composition of the Urine.*** After strenuous exercise the urine volume generally decreases, while the acidity and ammonia increase. The phosphate and lactic acid excretion increase and the chloride excretion decreases. The maximum effect usually is obtained in 20 minutes. Normal values should be restored in about an hour.

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[103] Dextrin-starch mixture may be prepared by boiling 200 g. of starch in 900 ml. of water, cooling to 50° C., adding a little malt extract, and allowing to digest until completely liquefied.

Procedure. **The subject who has had no food for several hours is given 50 ml. of water every 15 minutes throughout the experiment, beginning for example at 12:00 noon. At 12:00 the bladder is emptied and urine specimens collected every 15 minutes thereafter. At 1:00 the subject engages in strenuous exercise for 2–3 minutes. Running up and down stairs for this period will suffice. In the urine specimens determine lactic acid, phosphate, total acidity, chloride, ammonia, and pH. (For methods, see Chapter 31.) Plot curves of amounts of these constituents per 10-minute specimen against time to show the effect of exercise on urine composition. The blood sugar may also be determined at intervals and the influence of the exercise noted. Curves from a typical experiment are shown in Fig. 267.**

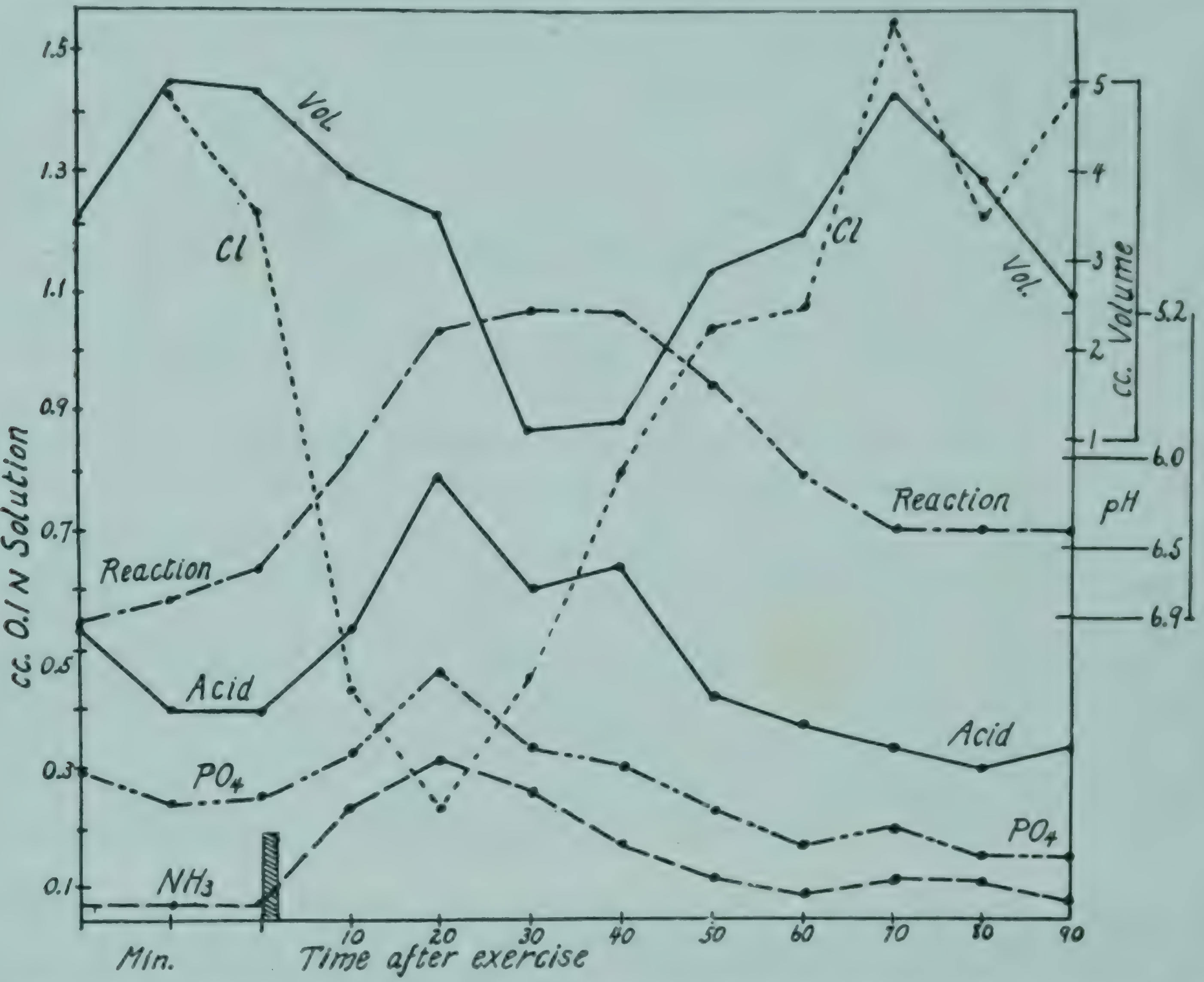

FIG. 267. CURVES SHOWING INFLUENCE OF EXERCISE ON COMPOSITION OF THE URINE.

Courtesy, Wilson, *et al.*: *J. Biol. Chem.*, 65, 755 (1925).

4. ***The Effect of Insulin on the Blood Sugar Level.*** See the experiment described on p. 769.

5. ***Influence of Carbohydrate Deficiency.*** Carbohydrates occupy a very prominent place in the diet of man. That they are not *essential* dietary constituents, at least for the white rat, may be shown by the following experiment.

Demonstration on Carbohydrate Deficiency. **Use young white rats as subjects, feeding one rat Diet 1 and another rat Diet 2 as given in the following table:**

CARBOHYDRATE DEFICIENCY DIET

	Diet 1 per cent	*Diet 2 per cent*
Casein	55	20
Butter fat	30	15
Lard	15	10
Starch or dextrin	0	55
Yeast, dried,[104] gram per day	0.4	0.4

Both rats will grow normally in spite of the practical absence of carbohydrates in Diet 1. In the case of man, the withdrawal of carbohydrates is followed by ketosis (see p. 1073). This ketosis is absent or much less pronounced in the case of the white rat.

6. Isolation and Determination of Liver Glycogen. This exercise serves to illustrate the conditions for the manufacture and storage of liver glycogen by the animal organism, to provide some liver glycogen for study, and to show how tissue glycogen is quantitatively determined.

Procedure. **Students may work in pairs. Prior to the experiment, transfer a 6-ml. portion of 30 per cent KOH to each of two 50-ml. centrifuge tubes, stopper the tubes with a rubber stopper, and weigh on an analytical balance. Suspend the tube on the balance by means of a copper wire fastened around the tube just below the pouring lip. Take care that the alkali does not come in contact with the rubber stopper.**

Each group of four students will be supplied with a rat. Half the animals dispensed will have been starved and the other half fed. Remove a rat from its cage gently to avoid exciting it. Stun it by a blow on the head and decapitate it quickly. Immediately remove the liver, weigh quickly to the nearest 0.1 g., and cut into two approximately equal portions, one for each pair of students. Mince the liver portion immediately and transfer samples of the minced liver to each of the two centrifuge tubes (0.7 to 1.0 g. samples from fed rats and 1 to 1.5 g. samples from starved rats). Stopper the tubes and weigh again. The difference between this weight and the original weight of the tube plus the KOH solution will give the weight of liver sample used. Remove the stoppers and place the tubes upright in a boiling water bath for 15 to 20 minutes, agitating the solution occasionally to ensure thorough disintegration.

At this point each pair of students should exchange one of the two tubes for one from another pair of students that represents a rat of opposite type. Thus each group will have one glycogen preparation from a fed rat and one from a fasted rat. Add 7 ml. 95 per cent alcohol to each tube, mix by tapping, and immerse in the water bath until boiling just begins (care must be taken to avoid loss by sudden foaming). Allow the tubes to cool at room temperature for about 2 hours. Centrifuge, decant and discard the supernatant fluid, drain and wash the precipitates twice with 5-ml. portions of 60 per cent alcohol by centrifuging, decanting, and draining as before. Expel the

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[104] The yeast is fed separately, 0.4 g. to each rat. It may be fed in the form of a powder or as tablets. The only carbohydrate present in Diet 1 is the very small amount in the dried yeast.

last traces of alcohol by immersing the tubes in boiling water just long enough to dry the glycogen. Compare the relative amount of glycogen obtained from the fed animal with that from the fasted animal. To each tube add 10 ml. distilled water and stir until a uniform suspension is obtained. Pipet 5 ml. of the suspension into a clean test tube, add 5 ml. 2 N sulfuric acid solution, insert a small funnel in the mouth of the test tube to minimize loss by evaporation, and heat in a boiling water bath for 3 to 4 hours to hydrolyze the glycogen. Cool, add a drop of phenol red indicator, and neutralize cautiously with 1N NaOH, with constant stirring; a volume of about 10 ml. should be required. Transfer the neutralized solution quantitatively to a 50-ml. or 100-ml. volumetric flask, dilute to volume with water, and mix. Analyze this solution for glucose by the Somogyi-Shaffer-Hartman method (p. 571), using the volume of aliquot that will contain not less than 0.1 mg. or more than 2.0 mg. of glucose, calculated on the assumption that the glycogen content of the liver from the fed animal is approximately 10 per cent on a wet weight basis, and that from the starved animal is 0.2 per cent (it may be zero). Calculate the liver glycogen content as grams of glucose per 100 g. of liver, and report results to the instructor. In your discussion of the experiment, briefly discuss the factors which influence glycogen storage and depletion. Use the remaining glycogen suspension which was not hydrolyzed for performance of the iodine test (p. 281) and the saliva test (p. 281).

V. EXPERIMENTS ON FAT METABOLISM

1. Fat Utilization. This may be determined in a manner entirely analogous to that used in the determination of protein utilization (see p. 1047). The fat may be determined by the Saxon method (see p. 454). It is claimed when moderate amounts of fat are fed that the fat of the feces is largely independent of the diet. Therefore, in order to secure accurate information regarding the utilization of food fat, it is said to be necessary to determine the fecal fat on a nonfat diet. There may possibly be a fat excretion from the intestine but much of the lipide of the feces is found in cellular structures (bacteria, epithelial cells, etc.). (See Chapter 21.)

2. Fat in Feces. A normal adult will digest and absorb at least 90 per cent of the fat in the diet when the amount ingested does not exceed 100 g. If the diet contains an excessive amount of fat, e.g., 300 g. per day, considerable appears in the feces. In pancreatic diseases and such conditions as are accompanied by a decrease in bile flow, the digestion and assimilation of fat is lessened.

Experiment. **(*a*) Ingest an ordinary mixed diet containing an average amount of fat per day, e.g., 75–100 g. Collect a stool and examine it microscopically as directed on p. 449. (*b*) Now ingest a diet containing an excessive quantity of fat, e.g., 300 g. per day. Separate the feces and subject a representative sample of the feces from the high fat diet to microscopical examination. (*c*) If it is desired, the fat may be extracted from some of the stool by applying the principle involved in the quantitative determination of fat in the Saxon method (see p. 454). Evaporate the ether extract and identify the fat in the residue by tests given in Chapter 3.**

3. Influence of Fat (Fatty Acid) Deficiency. Although it has often been demonstrated that fats are not essential constituents of the diet from the energy standpoint, Burr and Burr have shown the essential nature of certain unsaturated fatty acids (see discussion on p. 1004). The following experiment demonstrates the characteristics of a deficiency of essential fatty acids in the rat.

Experiment. Two groups of rats, weaned at three weeks, are placed in individual cages and fed the following diet: purified casein[105] 24, sucrose 72.1, salt mixture (McCollum-Davis, see Appendix) 3.9. Supplementing this diet, 0.65 g. of dried ether-extracted yeast upon which is dried 2 drops of an ether extract containing the nonsaponifiable fraction of 70 mg. cod-liver oil, is fed daily. To each rat in one group 10 drops of lard are given daily. Water is supplied ad lib., and if desired, the quantity consumed may be measured.

In about 70 to 90 days the symptoms of the deficiency will begin to appear in the fat-free group. The legs, especially the hind legs, become scaly and swollen, the tail becomes spotted and ridged and finally necrotic (Fig. 268), the hair on the back becomes filled with dandruff and tends to fall out, and degeneration of the kidneys sets in, as may be observed from the bloody urine. The renal lesions are the immediate cause of death. In the females, ovulation becomes irregular and finally ceases.

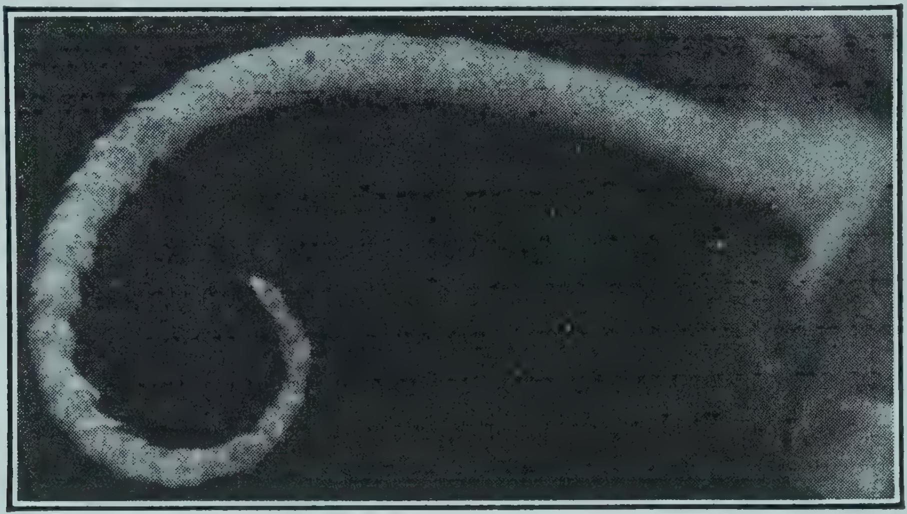

FIG. 268. NECROSIS OF THE TAIL OF A RAT ON A FAT-FREE DIET.
Courtesy, Burr and Burr: *J. Biol. Chem.*, **82**, 345 (1929).

The deficient animals eat the same amount of food but drink twice as much water as the controls. Before the deficient animals reach a moribund condition, their diet may be supplemented with a small amount of an oil or fatty food, to test the curative properties of the fatty acid molecules.

The above procedure can be employed quantitatively for the bioassay of the essential fatty acids present in foods.[106] Weanling male rats from litters of which the mothers have been on a low-fat diet are placed on a fat-free diet until depleted, as determined by a constant body weight for 3 weeks. This requires a period of approximately 12 weeks. The rats are then divided into the following groups: negative control group; groups to determine reference growth curve (which receive 10, 20, or 50 mg. methyl linoleate daily); groups to receive unknown fat at several levels (25 and 50 mg. per day for cottonseed and similar oils, 250 and 500 mg. per day for margarines and butter). The assay period is continued for 8 weeks. The potency of the unknown fat can then be estimated from the log dose: gain-in-weight relationship, as is done in the standard procedure for bioassay of vitamin A.

4. Ketosis. Ketosis may be induced in a normal person by the ingestion of a carbohydrate-free diet. The origin of ketosis is discussed on p. 1007. The intensity of ketosis may be evaluated by determination of the excretion of ketone bodies in the urine (ketonuria). The following table shows the data obtained in an actual case of the withdrawal of carbohydrate food from the diet of a normal man (Von Noorden).

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[105] Reprecipitated curd casein is washed until free from chlorides, extracted repeatedly with alcohol, and finally extracted for one week with ether, and dried at 35° C. For detailed directions see Burr and Burr: *J. Biol. Chem.*, **82**, 345 (1929).

[106] Deuel, Greenberg, Anisfeld, and Melnick: *J. Nutrition*, **45**, 535 (1951).

KETOSIS ACCOMPANYING CARBOHYDRATE WITHDRAWAL

Day	*Diet*	*Excretion of Acetone Bodies Calculated as β-Hydroxybutyric Acid*
		grams
1	Protein, fat, and *carbohydrate*	None
2	Protein and fat	0.8
3	Protein and fat	1.9
4	Protein and fat	8.7
5	Protein and fat	20.0
6	Protein, fat, and *carbohydrate*	2.2

Experiment. **Ingest an ordinary mixed diet for 1 day. Follow this by a period of 2 to 4 days in which no digestible carbohydrate is eaten. (A diet of meat, eggs, butter, agar-agar, and water has a very low digestible carbohydrate value.) Collect the urine for each day of the experiment, examine it qualitatively for acetone bodies (see tests, Chapter 29). If present, determine the total acetone bodies quantitatively (for methods see Chapter 31). The blood may also be examined (see Chapter 23). Did the withdrawal of carbohydrate food cause a ketonuria?**

VI. ADEQUATE vs. OPTIMAL NUTRITION; THE DIETARY EFFICIENCY OF MILK

The foregoing experiments have demonstrated that certain factors must be present in a diet if it is to be deemed *adequate*. But such a diet, although it qualifies under all required nutritional standards, may be improved upon. We thus form what may be termed an *optimal* diet. To demonstrate the difference between an *adequate* and an *optimal* diet proceed as follows:

Place two white rats from the same litter, each weighing 35–45 g., in separate cages. Feed one rat an *adequate* diet (see below) and the other an *optimal* diet (see below).

Adequate Diet		***Optimal Diet***	
Whole wheat	**82**	**Whole wheat**	**66**
Whole milk (powder)	**17**	**Whole milk (powder)**	**33**
Sodium chloride	**1**	**Sodium chloride**	**1**

It will be noted that dry milk constitutes only *one-sixth* of the adequate diet whereas it makes up *one-third* of the optimal diet. Give the animals water ad lib. and weigh them at least twice a week. Continue the experiment for at least 10 weeks, plotting the growth curve of each animal and keeping an accurate record of the food eaten (see Appendix for methods of recording data). If it is desired to investigate the relationship of these diets to reproduction, two rats of opposite sex may be used in each test and caged together.

The rat (or rats) receiving the *optimal* diet should grow more rapidly than the animal or animals receiving the *adequate* diet. If the experiment embraces the question of reproduction it will be found that the rats on the optimal diet have greater success in the rearing of young and that the offspring grow better during the nursing period than do the offspring of the rats ingesting the *adequate* diet.

In a comprehensive series of observations on these diets by Sherman and associates it has been found that the animals fed the optimal diet mature earlier and live longer. In common with other data secured from tests with albino rats these findings may be applied directly to the human dietary, and afford a very striking demonstration of *the remarkable nutritive efficiency of milk.*

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34

Inorganic Metabolism

Composition of the Animal Body. The elements that compose the human organism and their approximate relative amounts in the body are as follows:

	Per Cent	*Approximate Amount in a 70-Kg. Man*
		grams
Oxygen	65.0	45,500
Carbon	18.0	12,600
Hydrogen	10.0	7,000
Nitrogen	3.0	2,100
Calcium	1.5	1,050
Phosphorus	1.0	700
Potassium	0.35	245
Sulfur	0.25	175
Sodium	0.15	105
Chlorine	0.15	105
Magnesium	0.05	35
Iron	0.004	3
Manganese	0.0003	0.2
Copper	0.0002	0.1
Iodine	0.00004	0.03

Various other elements are also present in traces; these are sometimes called as a group the *trace elements*. In many instances their nutritional significance if any is obscure. In addition to copper, manganese, zinc, and iodine, which may be considered trace elements of known nutritional significance, this group includes the elements aluminum, fluorine, silicon, lithium, bromine, arsenic, lead, molybdenum, vanadium, and possibly others. Each of these elements is in fact a mixture of *isotopes*, elements that have identical chemical properties but differ in certain physical properties (see Chapter 32, "Isotopes").

The elements which make up the animal body are obtained from various sources. Carbon, hydrogen, and oxygen are supplied to the body by carbohydrates, fats, and proteins, and by water and the oxygen of the air; nitrogen, as well as a considerable proportion of phosphorus and sulfur, are supplied by proteins; the remaining elements are supplied by the mineral constituents of natural foodstuffs, by common salt, and to some extent by the minerals of drinking water.

About 90 per cent of the total oxygen of the animal body and about 70 per cent of the total hydrogen are present together in the form of water, which makes up roughly two-thirds of the total body weight. The remaining oxygen and hydrogen, along with all the nitrogen, most of the carbon and sulfur, and some of the phosphorus, are found in the organic compounds of the body (carbohydrates, lipides, proteins, etc.). These compounds comprise about 90 per cent of the total solid matter of the animal body, the remaining 10 per cent (about 3.3 per cent of the total body weight) being largely inorganic. The inorganic elements essential for

Proximate Chemical Composition of an Adult Human Body

Adapted from the data of Mitchell, Hamilton, Steggerda, and Bean (*J. Biol. Chem.*, **158**, 625 (1945)). The subject was an adult male, aged 35, estimated to have been in reasonably good nutritional state at the time of death, which was due to an acute heart attack. The body was obtained from the Department of Anatomy of the University of Illinois College of Medicine, and had been preserved only by freezing until the dissection for analysis was begun about 6 weeks after death. Approved analytical methods were used.

Parts Analyzed	*Per Cent of Total Body*	*Water*	*Ether Soluble Solids*	*Crude Protein (N × 6.25)*	*Ash*	*Calcium*	*Phosphorus*
		%	%	%	%	%	%
Skin	7.81	64.68	13.00	22.19	0.68	0.0205	0.060
Skeleton	14.84	31.81	17.18	18.93	28.91	11.02	4.83
Teeth	0.06	5.00*	..	23*	70.90	24.42	11.81
Striated muscle	31.56	79.52	3.35	16.50	0.93	0.0099	0.116
Brain, spinal cord, and nerve trunks	2.52	73.33	12.68	12.06	1.37	0.0188	0.352
Liver	3.41	71.46	10.35	16.19	0.88	0.0102	0.148
Heart†	0.69	73.69	9.26	15.88	0.80	0.0078	0.113
Lungs‡	4.15	83.74	1.54	13.38	0.95	0.0116	0.114
Spleen	0.19	78.69	1.19	17.81	1.13	0.0079	0.217
Kidneys	0.51	79.47	4.01	14.69	0.96	0.0130	0.174
Pancreas	0.16	73.08	13.08	12.69	0.93	0.0143	0.155
Alimentary tract	2.07	79.07	6.24	13.19	0.86	0.0125	0.115
Adipose tissue	13.63	50.09	42.44	7.06	0.51	0.0116	0.048
Remaining tissues							
Liquid	3.79	93.33	0.17	5.68	0.94	0.0054	0.066
Solid	13.63	70.40	12.39	16.06	1.01	0.0675	0.053
Contents of alimentary tract	0.80	..	..	..	..	..	..
Bile	0.15	..	..	..	..	..	..
Hair	0.03	..	..	..	..	..	..
Total body, weighing 70.55 kg.	100.00	67.85	12.51	14.39	4.84	1.596	0.771

* Assumed.

† Somewhat enlarged.

‡ Somewhat congested.

animal life include sodium, potassium, calcium, magnesium, phosphorus, iron, copper, chlorine, iodine, cobalt, manganese, zinc, and possibly others.

The relative amounts of water, organic material, and inorganic elements ("ash") characteristic of the body as a whole do not necessarily represent the composition of the individual tissues and organs of the body, where wide variation may be encountered, not only from tissue to tissue but for a particular tissue under varying normal and pathological conditions. These differences between the various parts of the animal body in this respect are illustrated by the data in the table on p. 1078.

The term "proximate" refers to an analysis in terms of fundamental components but not necessarily in terms of the elements themselves. Fresh tissues or tissues from different animal species may vary more or less from the values given in the table. The weight of the ash is slightly higher than the actual weight of mineral elements present, including as it must the carbon and oxygen of carbonates, the oxygen of phosphates and sulfates, etc.

The absence of carbohydrate from the table is to be noted. If the carbohydrate content is estimated by difference, it cannot account for more than a few per cent of the total body weight. This emphasizes the fact that in animal tissues as a whole, carbohydrate is present in limited amount, despite the high carbohydrate content of the animal diet—in contrast to plant tissues, where carbohydrate may make up the major portion of the solid matter present. Another factor which enters into the picture is that much of the carbohydrate in animal tissues readily undergoes post-mortem changes, i.e., glycogen and glucose are converted to lactic acid, etc., so that a precise estimation of carbohydrate content requires special technical treatment.

WATER

Water is the most abundant compound in the animal body, making up approximately two-thirds of the total body weight, although the various tissues within the body may differ significantly in water content (see table, p. 1078). Other organisms, e.g. the jelly-fish, may have a much higher proportion of water. Water furnishes what has been called "the aqueous milieu within which life processes occur." Water is more essential than food in the sense that an individual can survive much longer when deprived of food than when deprived of water. According to Thorpe, death usually results when about 20 per cent of the body water is lost. For experiments illustrating the need for water in the diet, see p. 1057.

Water possesses a number of physical and chemical properties which promote its physiological utility. The high solvent power of water permits the formation of a variety of true and colloidal solutions, within which reactions may occur at a much more rapid rate than if a solid phase were concerned. The high dielectric constant of water promotes ionization, thus facilitating reaction occurrence and velocity. Water itself enters into a great many of the reactions of biological material (hydrolysis, oxidation, reduction, etc.) and appears to catalyze other reactions in which it plays no apparent part. The lubricating action of water is important in connection with such physiological functions as swallowing and the role of the various internal fluids of the body (synovial, peritoneal, pleural, etc.).

Water is a good heat insulator, and at the same time appears to be of major importance in the control of heat loss from the animal body. The heat required to convert 1 g. of water to water vapor at room temperature is approximately 0.6 Cal. Du Bois found in one instance that the amount of water lost by vaporization from the skin and lungs of a normal resting man under ordinary conditions of temperature and humidity was about 680 g. per day. The heat loss associated with this vaporization represented about one-quarter of the total daily heat loss. Other means of heat loss include radiation and conduction; as the environmental temperature rises toward that of the animal body (37° C.), these latter means become relatively ineffective and heat loss by vaporization of water becomes proportionately greater.

The significance of water in the animal body[1] has two general aspects: (1) the relation between water entering the body and that leaving it, i.e. the water balance, and (2) the distribution of water between the various water-containing fluids of the body.

Water Balance. The sources of body water include (1) the fluids of the diet, (2) the solids of the diet, which contain more or less water, and (3) the water produced by the oxidation of material within the tissues. The major channels of water excretion include (1) the lungs, (2) the skin, (3) the kidneys (urine), (4) the intestinal canal (feces), (5) in the lactating female, the milk, and (6), to a minor extent, the tears. The relative significance of these various factors may be illustrated by the following table.

WATER BALANCE OF A NORMAL INDIVIDUAL

Source			*Excretion*		
	Ml.	*Per Cent of Total*		*Ml.*	*Per Cent of Total*
Fluids of diet	1200	48	Lungs	500	20
Food	1000	40	Skin	500	20
Oxidation within tissues	300	12	Urine	1400	56
			Feces	100	4
Totals	2500	100		2500	100

Normally an individual is in water equilibrium, i.e., water gain equals water loss with respect to all sources. A water intake in excess of the ability of the body to excrete water may be toxic (water intoxication); a water loss which exceeds water intake may lead to dehydration, and if sufficiently prolonged, to death.

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[1] For a review, see Abbott: *Am. J. Med. Sci*, **211**, 232 (1946). See also Hawk: "The relationship of water to certain life processes and more especially to nutrition," *Biochem. Bulletin*, **3**, 420 (1914); Hawk: "Water as a Dietary Constituent," in *Endocrinology and Metabolism* Vol. III, London and New York, D. Appleton and Co., 1924

Wide variation from the values given in the table is encountered under a variety of physiological and pathological conditions. Water intake by a normal person is largely a matter of individual choice or habit; it has been amply demonstrated that copious water drinking produces no harm and even if taken with meals promotes digestion owing to stimulation of glandular secretions. Water excretion through the skin as perspiration may be significantly increased at the expense of other channels, e.g. the urine, during severe exercise in a hot environment. Pathologically, the main channels of water loss, and the extent each may be involved, are as follows: by vomiting, up to 5 liters; overventilation, 1 to 2 liters; excessive perspiration, 10 to 15 liters; diarrhea, up to 5 liters. In diabetes insipidus, daily urine volumes of 30 liters or more have been observed, with a correspondingly large water intake.

Water excreted by the body through any channel except the lungs is invariably accompanied by electrolytes, chiefly sodium and chloride. The symptoms of excessive water loss (dehydration) are due to the loss of both water and electrolytes, and both water balance and electrolyte balance must be restored if the condition is to be alleviated. If, after prolonged profuse sweating, only water is restored to the body by drinking, neuromuscular spasms (cramps) may occur due to failure to replace the electrolytes lost in the sweat. Workers in a high-temperature environment and persons competing in athletic events have found that the addition of common salt to the drinking water will obviate this tendency.

Water excretion by the kidneys appears to be under the control of hormones from the posterior pituitary and the adrenal cortex. Absence or ineffectiveness of the "antidiuretic" principle of the posterior pituitary is believed to be the lesion in diabetes insipidus, although some balance with the anterior pituitary must be acting here, since the production of diabetes insipidus by experimental lesions of the posterior pituitary requires the presence of an intact anterior pituitary. The nerve fibers of the hypothalamic portion of the brain also appear to be of some importance in this connection. It is possible that, in the absence of the antidiuretic hormone, the tubular cells of the kidneys to a greater or lesser extent lose their ability to reabsorb the 99 per cent or more of the approximately 150 liters of glomerular filtrate produced in the kidney per day.

With regard to cortical hormones, of the pure principles thus far obtained (see Chapter 26), deoxycorticosterone is effective in maintaining normal water and electrolyte balance, as is the "amorphous fraction" of the cortical hormones. It is uncertain here whether the effect is primarily on the excretion of water or electrolytes, if indeed any distinction between these two can be made. This may also be true with respect to the pituitary principle. It serves to emphasize the fact that water excretion and electrolyte excretion are closely connected and should not be considered separately.

Distribution of Water in Body Fluids. The water of the body tissues and fluids is usually divided for consideration into two phases: (1) an extracellular phase, subdivided into (*a*) plasma (5 per cent of total body weight, or about 3 liters in a 70-kg. man), and (*b*) interstitial fluid, comprising about 15 per cent of the total body weight, or about 10 liters in an adult; and (2) an intracellular phase, making up about 50 per cent of the total body weight, or about 35 liters. Various methods, not all of which give the same result, have been used to establish this distribution of the body water and to follow variations in health and in disease. For example, a common basis for the determination of interstitial fluid volume is to determine the dilution of a known amount of injected material such as thiocyanate, sulfate, chloride, sucrose,[2] etc., which is assumed to distribute itself evenly throughout the extracellular water but not to penetrate the cells, at least under the experimental conditions. Suitable corrections for the amount present in the plasma, and the amount excreted, must of course be made. The use of heavy isotopes such as deuterium and tritium and of radioactive isotopes, e.g. radio-sodium, radio-chloride, and radio-sulfate, is of considerable value.[3] See Chapter 32.

The factors determining the distribution of water between the various fluid "compartments" of the body are not known with certainty, but they appear to be largely osmotic and related to electrolyte concentration, possibly modified by differences in permeability of ions to various membranes. The role of the plasma proteins in the water equilibrium between the blood and tissues has been discussed in Chapter 22. The role of inorganic ions may be visualized by the following necessarily inexact illustration. If it is assumed for example that the interstitial fluid contains sodium chloride in isotonic concentration, the presence of approximately 9 g. of sodium chloride will permit the retention of 1 liter of water, since sodium chloride in 0.9 per cent solution is isotonic. Loss of sodium chloride will necessitate an equivalent loss of water to keep the remaining fluid isotonic, and excess sodium chloride in the tissues will result in the presence of a larger volume of water for the same reason.

There appears to be an equilibrium between the various fluid compartments, to provide for the maintenance of a normal water content in the most essential phase. For example, the water content of the interstitial fluids may be decreased by 20 per cent or so without any detectable change in the water content of the plasma. Thus the water content of the plasma is not necessarily a reliable guide to general dehydration as some have assumed, at least in the early stages.

The inorganic compounds of the body exercise a great diversity of vital functions in the animal economy. The calcium phosphate–carbonate complex that forms the basis of osseous tissue gives to the bones and teeth their rigidity and hardness. Because of their influence on such phenomena as permeability of cell membranes, hydrogen-ion and osmotic equilibria, solubility of proteins, etc., the inorganic salts dissolved in the body fluids affect the contraction of muscle, the irritability of the nervous system, enzyme activities, water balance, and the secretion of digestive juices, perspiration, and urine. Certain of the inorganic elements, e.g. phosphorus, sulfur, iron, etc., are combined in the molecules of biologically important compounds such as glutathione, lecithin, hemoglobin and other proteins, etc.

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[2] Dean, Schreiner, and Robertson: *J. Clin. Invest.*, **30**, 1463 (1951).

[3] See Winkler, Elkington, and Eisenman: *Am. J. Physiol.*, **139**, 239 (1943); Walser: *Proc. Soc. Exp. Biol. Med.*, **79**, 372 (1952); Moore: *Science*, **104**, 157 (1946).

It has been demonstrated that inorganic elements must be available in the animal diet not only in adequate amount but also in proper balance with respect to one another. For example, a high intake of potassium salts results in increased elimination of sodium and chlorine, adequate dietary calcium is necessary for retention of potassium, and in cases of vitamin D deficiency, too high or too low ratios of calcium to phosphorus in the diet lead to serious impairment of bone structure.

In the following pages are considered some of the metabolic aspects of inorganic elements and compounds. Quantitative methods for the determination of various inorganic elements in blood and urine have been described in Chapters 23 and 31; the occurrence and significance of these elements in the urine are discussed in Chapter 28.

SODIUM

This element is present to a considerable extent in the diet and in the body fluids of the animal organism, invariably in the form of the sodium ion, Na^+, and in the diet at least, for the most part as sodium chloride. However, the sodium ion is a physiological entity whose function within certain limits is relatively independent of whatever anions (chloride, bicarbonate, lactate, phosphate, proteinate, etc.) are associated with it.

The adult human body contains about 100 g. sodium, distributed almost entirely in the extracellular fluids (blood plasma, interstitial fluid) of the body. On the average, about 4 to 5 g. sodium are ingested per day in the ordinary diet, corresponding to about 10 g. sodium chloride, which is the major form in which this element is ingested. Practically all of this ingested sodium is excreted by way of the urine. On diets low in sodium the urinary excretion falls to a very low level, so that the daily sodium requirement is considerably in excess of the amounts ordinarily ingested, and is commonly covered by the amount of sodium chloride (table salt) in the diet.

Carnivorous animals receive adequate salt from the animal tissues which constitute their food. The food of herbivorous animals is relatively low in sodium, and is high in potassium, the ingestion of which causes an increased urinary excretion of sodium. This loss of sodium induces a craving for this element, which in nature may be satisfied by such animals traveling long distances to salt licks, while domestic cattle are furnished with salt blocks. In the case of human subjects, the addition of salt to the diet makes it possible to eat with relish larger amounts of vegetable foods. Lactating cows not given a salt supplement yield milk of low sodium chloride content and may suffer otherwise. It has been shown in rat experiments that sodium deficiency unfavorably affects the appetite, the normal increase in weight, the storage of energy, and the synthesis of fat and protein. On a diet otherwise normal but of restricted sodium content (0.002 per cent), rats show retarded growth and disturbances of the eyes and reproductive function, with death ultimately ensuing. Rats may have their sodium needs satisfied by diets containing as little as 0.1 per cent of sodium chloride.

It has been shown by the use of radioactive sodium that this element is absorbed exceedingly rapidly from the gastrointestinal tract, some absorption apparently taking place even from the stomach—somewhat surprising in view of the relatively restricted absorptive capacities of this organ. Radioactive sodium (Na^{24}) has also been used as a means of determining the volume of extracellular fluid in the animal body, on the assumption (which appears to be reasonably valid) that the sodium ion is distributed evenly or in a predictable way throughout the extracellular fluids of the organism and is virtually absent from the body cells.

The major functions of the sodium ion in the animal body appear to be in connection with osmotic-pressure regulation and acid-base balance, although other possible functions such as a catalytic effect on enzyme activity, though as yet undiscovered, cannot be excluded. The role of sodium in osmotic pressure regulation and acid-base balance is best understood by a consideration of the electrolyte distribution of a typical body fluid (blood plasma) as shown herewith.

APPROXIMATE ELECTROLYTE DISTRIBUTION OF NORMAL HUMAN BLOOD PLASMA

	"Bases"					*"Acids"*			
Ion:	Na +	K +	Ca +	Mg	=	Cl +	HCO_3 +	Pr +	Others
Concentration: (milliequivalents per liter of plasma water)	154 +	5 +	5 +	3	=	106 +	28 +	17 +	16
				167	=	167			

The numerical values are to be considered as approximations only, subject to more or less wide variation under both normal and pathological conditions. As a matter of fact, the ionic concentration of an element in a biological fluid is somewhat difficult to define with exactness, since what is ordinarily measured is the total amount of element rather than that fraction which is ionized (see discussion of calcium, p. 1088). Nevertheless, the relationship shown above serves to bring out several fundamental facts.

In the first place, it must be true that the sum of all the cations present (Na, K, etc.) equals the sum of all the anions (Cl, HCO_3, etc.). This is evident only if concentrations are expressed in terms of milliequivalents rather than milligrams. This is one reason for the increasing use of the former term in connection with such substances as sodium, chloride, bicarbonate, etc., in their relation to physiological processes. For example, if bicarbonate is replaced by chloride in plasma, this replacement is on the basis of ion for ion (i.e., equivalent for equivalent) rather than gram for gram. The equivalent concentration of a monovalent ion may be obtained by dividing the weight of ion present by the equivalent (combining) weight of the ion; for milliequivalents, both weights are expressed in milligrams. Thus if sodium is present to the extent of 354 mg. per 100 ml., or 3,540 mg. per liter, the milliequivalent concentration per liter is 3,540/23 = 154 milliequivalents (mEq.). For a divalent ion such as Ca^{++}, the equivalent weight is the combining weight divided by 2, since one Ca ion is equivalent to two monovalent anions.

From the data on electrolyte distribution, therefore, it can be seen that the sodium ion is the chief cation of blood plasma, and this is true also for the other extracellular fluids of the body. Within the cells, sodium is present in relatively low concentration, being replaced largely by potassium and magnesium.

The division of the various ions of plasma into "bases" and "acids" deserves some clarification, since this usage is rare in general chemistry though quite common in physiology and medicine. The plasma (and the tissue fluids in general) are essentially neutral in reaction, and remain so under all conditions compatible with life. When an acid such as lactic acid enters into or is produced within a neutral buffered physiological fluid, to all intents and purposes the hydrogen ion of the acid disappears in the formation of water or other un-ionized molecule. The fluid still remains essentially neutral in reaction, and the only evidence that acid has been present is found in the increased amount of the acid anion—e.g., the lactate ion in this instance. This increased acid anion content is at the expense of such buffer anions as bicarbonate, phosphate, proteinate, etc., originally present. Thus the entrance of acid is reflected not by any marked change in pH, but by alteration in the relative amount and distribution of the various anions present. The anions therefore represent the "acid" portion of the electrolytes. An analogous situation prevails with respect to base, the entrance of which, within the framework of a neutral solution, will be evidenced almost entirely by an increase in cation (e.g. Na) content. Naturally, other factors enter into acid-base balance within the body, as is evident from the discussion in Chapter 24, but the concept of acid-base balance reflected in part by the kind and distribution of anions (acids) and cations (bases), within an essentially neutral solution, is of considerable value in an understanding of all phases of this subject. From what has been said, it can be seen that sodium is the chief base of the plasma, and of the extracellular fluids in general.

Another aspect of sodium-ion concentration which is of importance is in connection with osmotic pressure regulation. The total osmotic pressure of a biological fluid such as the blood plasma is equal to the sum of the osmotic effectiveness of all the ions present. Thus the plasma corresponds osmotically to a solution 0.167 M (167 milliequivalents per liter of plasma water) with respect to an electrolyte such as NaCl, which is assumed to be completely dissociated in solution to yield two ions, or to a solution of a nonelectrolyte which is approximately 0.33 M (2 × 0.167). The *osmolar* concentration of plasma is therefore approximately 0.33. Note that of the total osmotically effective bases of plasma, sodium makes up approximately 92 per cent ($^{154}/_{167}$). Thus changes in the osmotic pressure of plasma (and in general of the extracellular fluids of the body) are largely due to, and may be caused by, changes in the concentration of sodium present. It is true that under stress a loss of sodium may be compensated for to a small extent by an equivalent increase in potassium, but the ability of the organism to substitute *bases* in this manner is relatively restricted (in contrast to the wide variation in anion distribution which may occur) and any major loss of sodium from the body, as in Addison's disease, and prolonged diabetic ketosis, leads to a significant lowering of the osmotic pressure of the body fluids, and therefore water loss (dehydration). Restoration is not complete until both the lost base and the lost water are replaced.

The role of sodium in acid-base balance is secondary to its role in maintaining the total osmotic pressure of the body fluids. That portion of the body sodium equivalent to the bicarbonate present (i.e., about 28 milliequivalents per liter of plasma water) represents most of the available base of the plasma which can be used for the neutralization of entering acids, and in conjunction with carbonic acid determines the pH of blood (see Chapter 24). It is more correct however, to regard acid-base changes in terms of the anions present rather than in terms of sodium. Thus one may have an acidosis or alkalosis in terms of variation in plasma bicarbonate and CO_2 tension without significant changes in sodium content.

The sodium ion has an effect on irritable tissues such as muscle which does not appear to be related to osmotic forces, and which is counteracted by the presence of the calcium ion, in the proportion of about 1 to 2 calcium ions per 100 sodium ions. This is the basis for the use of such "physiologically balanced" solutions as Ringer's, Tyrode's, etc., rather than isotonic saline alone, in experiments involving the maintenance of function of isolated animal tissues or organs.

The excretion of sodium has been discussed on p. 963. On an ordinary diet urinary sodium amounts to about 3 to 5 g. per day, corresponding to 10 to 12 g. expressed as sodium chloride, but wide variations naturally can be expected. On a low-salt diet and in starvation, urinary sodium chloride excretion falls to very low levels. The activity of the antidiuretic hormone of the posterior pituitary (see p. 775) is sensitive to the availability of corticosterone and deoxycorticosterone, elaborated by the adrenal cortex. As a result, a deficiency of these steroid hormones is followed by a loss of sodium and chloride in the urine and retention of potassium, although the causative relationship has been questioned.[4] The restriction of dietary sodium chloride is widely advocated in essential hypertension and cardiac disease.

CHLORINE

This element is found in biological material exclusively in the form of the chloride ion;[4a] the biological significance of postulated organic chlorine compounds, such as chlorolipides, has as yet to be conclusively demonstrated. In the diet chloride is found largely as sodium chloride, the daily intake approximating 6 to 7 g., representing about 10 g. of sodium chloride, but wide variation in this respect is encountered, depending as it does upon dietary habits. The adult human body contains approximately 100 g. of chloride, found largely in the extracellular fluids of the body, but present to some extent in red blood cells and to a lesser extent in the other cells of the tissues.

Although a close relationship exists between chloride and sodium in certain physiological processes, the chloride ion has a number of functions which are peculiar to it and which are essentially independent of sodium ion functions. Thus it is the chief anion of the gastric juice, being present there in approximately the same concentration as in the blood, at least before dilution or other modification of the gastric juice, and accompanied by the hydrogen ion in substantially equivalent amount, rather than by sodium as in the plasma and extracellular fluids of the body. The chloride of gastric juice is apparently derived ultimately from blood chloride (see Chapter 14), and is normally reabsorbed during later stages of digestion. Loss of gastric juice chloride by vomiting, or in pyloric or duodenal obstruction, may lead to the development of an alkalosis due to bicarbonate excess, since the lost chloride is replaced in part at least by bicarbonate. It is interesting to note that on chloride-deficient diets there is no change in the output of chloride in the gastric juice, while chloride excretion by other channels, such as the urine and the perspiration, may be markedly decreased.

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[4] Fourman, Reifenstein, Kepler, Dempsey, Bartter, and Albright: *Metabolism*, 1, 259 (1952).

[4a] A notable exception to this rule is the antibiotic Chloromycetin(chloramphenicol), which contains chlorine in organic combination.

Another specific function of the chloride ion is in connection with the *chloride shift* in the blood during the carriage of carbon dioxide (see Chapter 24) whereby the bicarbonate content of the blood plasma is significantly increased by exchange with plasma chloride, which enters the red cells.

Chloride is likewise concerned in osmotic pressure regulation, making up about two-thirds of the total anions of blood plasma and occupying a similar position in the other extracellular fluids of the body. The role of chloride in this connection, however, is subject to somewhat greater variation than the role of sodium, since to a certain extent the organism can exchange chloride ions for other anions within the framework of a normal osmolar concentration of total anions. This ability to replace chloride ions by other ions is more restricted, however, than for the other major anion of the plasma, the bicarbonate ion, over half of which can be replaced, for example by the lactate ion under certain conditions.

The chloride ion is an activator of salivary amylase, but this action is not specific for chloride and may be due in part to the effect of strong electrolytes in general on the solubilities of such proteins as globulins. Radioactive chloride (Cl^{38}) has been used for studies on the volume of the interstitial extracellular fluid of the body.

Chloride present in the diet in excess over that required by the body is excreted, chiefly by way of the kidneys. Excreted chloride usually is accompanied by excess sodium or potassium unless there exists a need for conserving base, in which case the ammonium ion accompanies the chloride ion to a variable extent. Factors controlling the excretion of chloride are similar to those concerned with the excretion of sodium and potassium. On a chloride-deficient diet, or in fasting, the chloride excretion may amount to but a trace. However the excretion by normal adults averages about 10 to 15 g. per day expressed as sodium chloride.

POTASSIUM

This element, like sodium and chlorine, is also present in plant and animal tissues entirely as the potassium ion. The adult human body contains about 250 g. potassium, present almost entirely in the cells rather than in the extracellular fluids as with sodium. While normal human blood plasma contains only about 5 milliequivalents of potassium per liter of water, almost the entire cation content of red cells is made up of potassium (about 170 milliequivalents per liter of cell water). Thus potassium is the major "base" of the body cells, and apparently subserves in the cells the same general functions relating to osmotic pressure regulation and acid-base balance that have already been described for sodium in the extracellular fluids of the body. This is not the only function of potassium, however; it has been shown to aid in the enzymatic transfer of phosphate from ATP to pyruvic acid, for example (see p. 994); and the toxic effects of a significantly elevated plasma potassium level (potassium poisoning) can hardly be ascribed to osmotic forces alone. The potassium ion has an effect on muscular irritability which, like that of sodium, tends to antagonize the effect of the calcium ion. Nevertheless, under conditions of severe dietary salt restriction, calcium appears highly important in supporting the potassium content of tissue.[5]

The abundance of potassium in plant foods and in meat precludes the danger of a deficiency of this element in a mixed diet. From 1 to 3 g. is commonly excreted in the urine per day on such a diet. Growth in rats may be retarded by reducing the daily potassium allowance below 15 mg. in the male and 8 mg. in the female; this difference may be due to differences in growth rate. Potassium requirement varies considerably in the different animal species. Prolonged consumption of a potassium-deficient diet may result in failure of the animal to respond to a correction of the deficiency. Potassium deficiency in man is associated with weakness and muscular paralysis, accompanied by a fall in the plasma potassium level; in animals hypertrophy of the heart and kidneys has been noted. In Addison's disease the excretion of potassium falls and plasma potassium rises, with the reverse changes in urinary and plasma sodium, indicating a function of the adrenal cortex in this connection. The administration of sodium chloride alone will sometimes alleviate the symptoms of the disease, apparently permitting the organism to restore the altered relation between sodium and potassium retention and excretion. It has been claimed that the symptoms associated with removal of the adrenal cortex may be reproduced experimentally by measures which increase the plasma potassium level to about twice the normal value.

CALCIUM

Calcium is an essential constituent of all living cells. Its mode of action is not clear but it appears to play a part in decreasing the permeability of cell membranes and the irritability of cells in general. Its effect on neuromuscular mechanisms is shown in higher animals by the development of hyperirritability and tetany as a result of a decline in the calcium content of the blood. Such a fall is observed in tetany following removal of the parathyroids, symptoms becoming noticeable when the calcium-ion concentration falls below 3 mg. per 100 ml. (total calcium below 7 mg.).

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[5] Eppright and Smith: *J. Biol. Chem.*, **118**, 679 (1937).

Normal values are 9 to 11 mg. total or 4.25 to 5.25 mg. of ionized calcium. In fatal hyperparathyroidism, Ca^{++} values of 7 mg. (total calcium 16 mg.) have been noted. McLean and Hastings find the calcium of plasma nearly all accounted for as calcium ions and calcium bound with protein, an equilibrium existing between the two forms. The parathyroids appear of prime importance in regulating the calcium content of the blood. The mechanism of this action is not clear.

Vitamin D improves the utilization of calcium for calcification and other purposes. There is convincing evidence that vitamin D promotes calcium absorption; there is also some evidence that it may act on the calcification process itself. The mechanism of vitamin D action is obscure. Any relation between vitamin D and parathyroid action is, at most, indirect. The action of vitamin D in calcium metabolism is discussed further in Chapter 35. Since calcium salts within the intestinal tract are more soluble at acid reaction than in neutral or alkaline solution, absorption of calcium is promoted by high gastric acidities, by acid-containing diets in general, and by diets containing lactose which lead to lactic acid fermentation in the intestines. Amino acid products of protein digestion may promote the absorption of calcium, possibly by the formation of soluble complexes. Other as yet unknown factors may also be concerned.

We are also much in the dark as to the nature of the calcification process as it occurs in bones and teeth. It appears to depend upon an adequate inorganic phosphate concentration of the blood plasma as well as upon the Ca^{++} of the plasma, and these are maintained through a balance between absorption from the intestines and excretion by the intestines and the kidneys. One view is that we have a simple chemical or physicochemical equilibrium between blood and bone, the blood being commonly supersaturated with respect to calcium phosphate and carbonate, with cartilage possessing a certain selective action. Another view is that calcification involves an active chemical process in the bone cells, such as the liberation of inorganic phosphate from organic phosphates through the action of phosphatase. The subject of calcification is discussed further in Chapter 9.

Calcium Requirement. The recommendation of the National Research Council is 0.8 g. per day for adults. There is a rather wide zone of calcium intakes over which different individuals can maintain or approximate equilibrium; adaptation to lower intakes has also been demonstrated. The question has even been raised whether or not the adult human male requires dietary calcium.[6] Sherman found 16 per cent of American dietaries to fall below 0.45 g. The growing child requires considerably more calcium than the adult. Similarly increased amounts are needed by women during pregnancy and lactation. Otherwise a negative balance for calcium may result and the drain upon the calcium of the bones of the mother becomes excessive. Because of the large store of calcium in the bones the adult does not suffer so soon from calcium deprivation as the child. A lactating cow may lose as much as 20 per cent of its total supply of calcium without seriously interfering with milk production. In the growing child, however, any interference with the calcium supply leads to impaired calcification of the bones, as in rickets, as well as to slower growth of the body in general. It must be borne in mind that the child at birth is calcium-poor, i.e., the bones are incompletely calcified. The greater flexibility of the bones facilitates delivery, but the need of the newborn for calcium is necessarily more critical on this account.

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[6] Hegsted, Moscoso, and Callazos: *J. Nutrition*, **46**, 181 (1952).

Milk is of supreme importance in maintaining a proper calcium level in the diet. Every child should receive along with other foods a quart of milk (containing about 1.1 g. calcium) each day. Milk is recommended in supplying calcium, because of its high content of calcium, the available form in which this is present (utilization about 30 per cent) and because milk has otherwise a high nutritive value. Milk contains about 120 mg. of calcium per 100 g., cottage cheese contains about 100 mg. Of other foods, eggs are useful, containing 70 mg. per cent, mainly in the yolk; as are also green vegetables, cabbage or lettuce supplying about 45 mg. per cent of calcium. Some green vegetables, like spinach, though high in calcium, are not so satisfactory, since much of their calcium exists as oxalate and is not readily utilized by the body. In certain foods, such as some cereals, the presence of phytic acid (inositol hexaphosphoric acid) impedes calcium and magnesium absorption, since this substance forms an insoluble compound with calcium and magnesium. This effect can be readily overcome by adding calcium carbonate to the diet, as for example by incorporation into bread. Bread has about 30 mg. per cent of calcium, and meat on the average about 10. The utilization of calcium is improved by vitamin D, an adequate supply of which should therefore be insured.

The subject of calcium excretion has been discussed in Chapter 28. Most of the excreted calcium is found in the feces. This fecal calcium presumably represents unabsorbed dietary calcium. There is an intestinal excretion of calcium which may amount to 0.5 g. or more per day; most if not all of this appears to be reabsorbed. The many factors which influence the absorption and excretion of calcium have led some to doubt the validity of calcium balance studies as an index to calcium requirement unless these various factors can be subjected to rigorous control.

The urinary output of calcium depends upon the nature of the diet but averages about 0.1 to 0.3 g. per day for normal adults.

PHOSPHORUS

Since the greater part of the phosphorus of the body is associated with calcium in bone, the metabolism of these two elements is to a considerable extent parallel and follows that of the osseous tissues. Phosphorus, however, is abundant also in many of the softer tissues of the body and plays many important roles in life processes. Through the intermediary formation of lecithins it is concerned with fat metabolism. Through the formation of hexosephosphates, of adenylic acid, and of creatine phosphate, it plays a primary role in the carbohydrate metabolism of animals as well as in fermentation processes. Phosphates play a role in the neutrality regulation of the organism (see p. 818). They are concerned with the absorption of sugars from the intestine and the reabsorption of glucose in the kidney tubules. Phosphorus is a constituent of the phospholipides present in all tissues and which are especially abundant in nervous tissues. It is present in nucleoproteins of the chromatin material of cells and in phosphoproteins such as casein. It has been suggested that most of the phosphorus, as well as the fat of milk, arises from the lecithin of the blood of the lactating animal.

Many studies of phosphorus metabolism have been made using radioactive phosphorus; this isotope also has some use in the treatment of disease (see p. 983). The important conclusions regarding phosphorus in its relation to bone and teeth formation which have resulted from the use of radioactive phosphorus have been presented in Chapter 9. For a further discussion of phosphorus in its relation to calcium metabolism and vitamin D, see Chapter 35.

Phosphorus Requirement. The National Research Council states that the daily phosphorus allowance for children and for women during pregnancy and lactation should be fully equal to the calcium allowance. The allowance for other adults should be 50 per cent higher than the calcium allowance (see Table, p. 1108). Four per cent of American dietaries have been found to fall below the minimum phosphorus requirement, the danger of deficiency being less than in the case of calcium, and yet a matter of some concern. The phosphorus content of the diet is most readily maintained at a proper level through the liberal use of milk, a quart of milk a day containing 0.88 g. of phosphorus being recommended for children. In addition to milk (93 mg. of phosphorus per 100 g.) good sources of phosphorus are meat (average 175 mg. per cent), eggs (180 mg.), cheddar cheese (610 mg.), nuts (400 mg.), whole cereals (as whole wheat, 375 mg.), while white flour and polished rice are much lower in phosphorus (about 90 mg.). The phosphorus in the bran of cereals (phytic acid) is utilized with some difficulty but, in general, the form of phosphorus in the diet is of little practical importance since in any case, as the result of the digestive processes, the phosphorus is absorbed in inorganic form. Too high a ratio of calcium to phosphorus in the diet, however, is unfavorable to phosphorus absorption. A diet high in beryllium or strontium also hinders phosphorus absorption and gives rise to a form of rickets.

MAGNESIUM

Magnesium is an indispensable constituent of all living cells. The daily requirement for magnesium has been estimated to be about 0.43 g. for adults. Magnesium is present in such amount in plant foods and in meat that there is little possibility of the diet being inadequate with regard to this element.

About 71 per cent of the magnesium of the body is present in the bones. In muscle magnesium exceeds calcium in amount. In blood, where the reverse is true, the magnesium content is very constant. This is true also of the body as a whole, the concentration of magnesium remaining constant at about 0.045 per cent during growth, whereas calcium and phosphorus tend to increase in percentage. Excessive intake of magnesium gives rise to an increased excretion of calcium in the urine, and vice versa.

Like calcium, magnesium depresses nervous irritability, but to a greater extent. A level of magnesium in the blood of 20 mg. per cent produces anesthesia, apparently by action on the central nervous system, whereas injections of calcium salts have a wakening effect. Magnesium ions are necessary for the action of many enzyme systems, particularly those concerned in carbohydrate and fat metabolism in muscle and other tissues. The mode of action of the magnesium ion in these instances is not known. Magnesium ions inhibit the activity of adenosinetriphosphatase, the enzyme which splits phosphate from ATP. It has been suggested that this may be related to magnesium anesthesia.

Diets extremely low in magnesium (1.8 parts per million) have been fed to young rats; under these conditions serum magnesium drops to 0.5 mg. per cent and there is marked vasodilation and hyperexcitability, leading to tetany and death. There is a rise of cholesterol of the blood and a corresponding decrease in free fatty acids, perhaps representing a disturbed metabolism of fat. There is also a marked decalcification of the skeleton which recalls the fact that grass tetany of cattle is associated with a low magnesium content of the blood.

Intestinal absorption of magnesium salts does not present a nutritional problem because of the relative solubility of the magnesium salts and their abundance in the diet. The excretion of magnesium has been discussed in Chapter 28. The action of magnesium sulfate in the duodenum in bringing about emptying of the gallbladder is employed in clinical diagnosis and therapy.

IRON

Iron exists in the body chiefly in the heme portion of the hemoglobin of the red blood cells. It is also found, however, in plasma in combination with serum globulin and as an indispensable constituent of various oxidation-reduction enzymes essential for the life of cells in general. Among the heme-containing enzymes are catalase, peroxidase, the cytochromes and cytochrome oxidase, and probably others. The action of these enzymes is inhibited by cyanide, which apparently combines with the iron. Iron is also found in the liver in the form of a compound called ferritin. Ferritin is an iron-containing protein, the protein portion of which has a molecular weight of about 500,000, and the iron is considered to be present in the form of colloidal $Fe(OH)_3$ interspaced in the crystal lattice of the protein. The full significance of ferritin in nutrition remains to be elucidated.

Young animals (of all species so far studied) placed on a pure milk diet at the time of weaning develop a severe anemia. Addition of purified iron salts alone to the diet does not cure the condition, but does so if supplemented with very small amounts of copper salts. It is well established that the animal organism cannot convert the iron of the diet into hemoglobin unless very small amounts of copper are also present. There are a few instances in the literature where this has also been demonstrated in the case of human nutritional anemia. In the majority of human nutritional anemias, however, a copper deficiency does not exist and the anemia is due simply to iron deficiency. These nutritional anemias are not to be confused with the anemia of folic acid deficiency (see Chapter 35) or with pernicious anemia or other anemias due to increased blood destruction or disturbances of the blood-forming organs.

Iron Requirement. The recommended daily allowance of iron as given by the National Research Council is 12 mg. per day for adults (15 mg. per day in pregnancy), and 6 to 15 mg. per day for children, depending upon age. It has been estimated that for infants 0.5 mg. per kg. body weight per day is necessary to insure retention of iron. The form of the iron in the diet must also be considered. Anemia due to iron deficiency is not uncommon in women and preschool children. Hence general adequacy of this element in the diet cannot be assumed. Infants are born with a high level of hemoglobin iron and with an additional iron reserve in their livers which is later used for hemoglobin formation to supplement the low iron content of their milk diet. If continued beyond the normal lactation period such a diet leads to the development of a nutritional anemia. Children ordinarily require supplementary iron-containing foods by the time they are one year old. There is some evidence that the administration of iron to normal children and young adults, or increasing the iron intake above the usual levels by other means, will consistently produce a slight but significant (about 10 per cent) increase in the blood hemoglobin content.

Milk contains from 0.07 to 0.1 mg. iron per 100 g. This small amount of iron appears to exist in a readily assimilable form. Most foods appear to contain two forms of iron: heme iron which is not utilizable and nonheme forms which are available. Metallic iron or any of the ordinary iron salts may be used to supply iron. It has been shown[7] that under certain conditions ferrous compounds are somewhat better utilized than ferric compounds. Eggs contain 3 mg. iron per 100 g. of food, all of the iron being available. Lean muscle meats contain about the same amount, 50 per cent of which is available. Beef liver or heart contain about 8 mg. per cent of iron, about 60 per cent of which is available. Spinach contains about 3.6 mg. iron but like the iron of blood, alfalfa, and oysters this appears to be less than 25 per cent available. The minimum amount of iron which must be added to milk for maximum hemoglobin regeneration is said to be 0.0007 per cent.

The absorption and excretion of iron have been the object of much study. Relatively little is known about the mechanism of iron absorption, but it is known for example that the action of copper in promoting iron utilization is not related to iron absorption. There is some indication that the absorption of iron may be influenced by the state of the body iron stores. Studies with radioactive iron have shown that iron may be absorbed very rapidly, appearing in the red blood cells in about four hours. However it takes about a week for the complete conversion of absorbed iron into hemoglobin.

Very little iron is ever excreted. Only 1 to 3 mg. per day is found in the urine, with more in the feces, but this latter probably represents for the most part unabsorbed iron of the diet. Iron split off from the hemoglobin of the red cells after their destruction appears to be retained within the body and used over again. The chief need for iron appears to arise in infants on a milk diet, or in others after blood loss as by hemorrhage or during menstruation.

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[7] Moore, Dubach, Minnich, and Roberts: *J. Clin. Invest.*, **23**, 755 (1944).

COPPER

Although copper has long been known as a constituent of such compounds as the hemocyanin of the blood of certain lower organisms, the first demonstration that copper played a vital part in the animal organism was in connection with the conversion of dietary iron to hemoglobin, as described on p. 1092. Since that time, copper has been shown to be an essential component of certain enzymes such as tyrosinase and ascorbic acid oxidase, and a copper-containing protein (hemocuprein) has been isolated from animal blood. Animals placed on a copper-deficient diet soon lose weight and die, but death is not due to the concomitant anemia, since an equally intense anemia due to simple iron deficiency may be maintained for a long time. Thus copper is an essential element in the animal body, but the precise role still remains obscure.[8] Some studies have demonstrated that copper is associated with the activity of certain oxidation-reduction enzymes in tissues.

The amounts of copper required per day are extremely small; for an adult only about 1 to 2 mg., whereas a child requires only 0.05 mg. per kg. of body weight. The adult human body contains about 0.1 g. of copper. Copper appears generally to be present in the ordinary mixed diet in adequate amounts, so that the possibility of a copper deficiency is limited. Perhaps because of this fact, there have been relatively few demonstrations of copper deficiency in man.

IODINE

The adult human body contains about 25 mg. iodine, about 15 mg. of which is in the thyroid. The daily allowance recommended by the National Research Council is about 0.003 mg. per kg. body weight. It is needed for the production of thyroxine and a deficiency leads to the condition of simple goiter. Ocean water is relatively rich in iodine, as is also the dust formed in the atmosphere from the drying of the salt spray, which dust is carried inland by the winds, where the rains dissolve it and carry it into the soil which thus becomes enriched with iodine. Where the distances from the sea are greater or mountain barriers intervene, this does not occur; at the same time iodine is being leached out of the soil, which thus becomes low in iodine. In such regions, including the Great Lakes region and the Alpine regions of Europe, the vegetable foods grown in the iodine-poor soil are poor in iodine, as is also the drinking water, and goiter is prevalent. In Japan goiter is very rare, in part because of the use in the diet of seaweed which is very high in iodine. Sea foods such as fish and oysters are rich in iodine. Good sources are also vegetables from sea coast districts. Fair amounts are also found in the fat of milk. In goiter regions, however, such foods should not be depended upon but should be supplemented with potassium iodide. A few drops of 10 per cent iodide solution given every two weeks would cover the requirement, since some storage of ingested iodine occurs. The most satisfactory method for insuring an adequate intake of iodine without danger of overdosage is to use table salt to which has been added one part per 100,000 of iodine as sodium iodide. Such salt is widely sold. Care must be taken in the administration of iodine to persons with hyperactive thyroids. Studies with radioactive iodine indicate that this element is rapidly taken up from the blood by the thyroid gland, with significant differences in this respect between normal, hyperthyroid, and hypothyroid individuals (see also p. 983). Iodides also appear to be selectively concentrated and excreted by the salivary glands in man.

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[8] Glass: *Copper Metabolism*, Baltimore, Johns Hopkins University Press, 1950; Marston: *Physiol. Revs.*, **32**, 66 (1952).

Variations in the iodine content of the blood show a relation to thyroid disease, but the results obtained are somewhat irregular. The normal range of serum iodine content is from 5 to 12 μg. per 100 ml. About 85 per cent of the serum iodine is bound to proteins, chiefly albumin. This constitutes the *protein-bound* iodine of the serum, the concentration of which is of diagnostic value regarding the level of thyroid function.[9] The normal level of serum protein-bound iodine is from 4 to 8 μg. per cent. A thyroid hyperplasia has been produced in rabbits by feeding cabbage. Various synthetic compounds are known, such as thiouracil and thiourea (see pp. 771–2) which exert a specific depressing effect on thyroid secretion and have proved useful in the treatment of thyroid disease.

SULFUR

Strictly speaking the metabolism of sulfur does not come under the head of inorganic metabolism, since only an insignificant part of the sulfur ingested is in inorganic form, by far the greatest proportion being combined in protein molecules as the amino acids cystine and methionine. Proteins of foods vary in sulfur content from 0.4 to 1.6 per cent, with an average of about 1 per cent. The sulfur-containing amino acids and their metabolism are discussed in Chapter 33.

The end product of sulfur metabolism is taurine or sulfuric acid; the latter is either immediately neutralized and excreted as inorganic sulfate in the urine or may be first conjugated with phenol, glucuronic acid, or indoxyl. On the average, about 1.0 g. sulfur is excreted daily in the urine.

Certain sulfur compounds have important biological interest, e.g., thiocyanate in saliva and other fluids, taurocholic acid in bile, ergothioneine of the blood corpuscles, and glutathione present in all cells and concerned with oxidation processes. These substances are discussed elsewhere in this book, as are also the properties and metabolism of the sulfur-containing amino acids and the various forms of urinary sulfur.

MANGANESE

There is considerable evidence that manganese in small amounts is a dietary essential. Rats placed on a manganese-deficient diet show a retardation of growth and reproductive failure in both male and female. The arginase activity of the liver of the manganese-deficient rat is lower than normal; *in vitro* addition of manganese ions restores the enzymatic activity. Other isolated enzyme systems are known to be activated by manganese ions in small amount. In the case of the growing chick, a manganese deficiency is associated with the development of perosis (bone malformation). Studies with radioactive manganese indicate that the chief channel of excretion is through the liver into the bile. It has been suggested that the daily diet of children should provide from 0.2 to 0.3 mg. manganese per kg. of body weight.

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[9] Salter, Bassett, and Sappington: *Am. J. Med. Sci.*, **202**, 527 (1941).

OTHER ELEMENTS

Fluorine. Fluorine is found widely distributed in animal and plant tissues, in very small concentration. When added to the diet in small amount, it is excreted in proportion to the intake. Individuals on a normal diet containing no added fluorine excrete about 0.3 to 0.5 mg. per day; this presumably represents the amount ordinarily present in the diet. In certain parts of the world the soil contains sufficient soluble fluoride so that the drinking water, and food grown on the soil, contain enhanced amounts of fluorine relative to other localities. Individuals living in such communities are prone to develop "mottled enamel" (dental fluorosis), an unsightly condition of the teeth. At the same time, the incidence of dental caries in these communities is in general much lower than elsewhere. The concentration of fluorine in water claimed to be effective in the prevention of caries, approximately 1 p.p.m., is said to be below the level which will cause dental fluorosis. A fluorine level of 6 p.p.m. apparently has no harmful effects on the bones. The fluoride ion in significant concentration (0.01 M) is an enzymatic poison for tissues, and fluoride salts are used commercially as ingredients of animal poisons. (For a further discussion of fluorine, see Chapter 9.)

Cobalt. Cobalt is present in plant and animal tissues in small amount, and there is conclusive evidence that it is an essential element in animal nutrition. It is present in vitamin B_{12} (see p. 1209). Certain diseases of cattle and sheep have been attributed to a cobalt deficiency, and respond to cobalt therapy. In cattle, cobalt dietary deficiency is accompanied by a microcytic hypochromic anemia, rapid loss of weight, absence of estrus, abortion, and low milk yield. In the case of sheep disease the cobalt is effective orally but not when injected. It has been shown that cobalt increases the synthesis of vitamins B_6 and B_{12} in the rumen of sheep. The administration of cobalt salts to rats and rabbits produces a marked polycythemia; the reason is not known. Studies with radioactive cobalt indicate that absorption and elimination take place rapidly, with the kidney the main channel of excretion.

Zinc. There is definite evidence that zinc is an essential element in animal nutrition. Rats on a diet low in zinc show a marked delay in intestinal absorption and a retarded growth rate. Zinc is a constituent of highly purified carbonic anhydrase, the enzyme important in the formation and decomposition of carbonic acid (see pp. 362 and 685). The zinc content of carbonic anhydrase is about 0.2 to 0.3 per cent, i.e., very similar to the iron content of hemoglobin (0.34 per cent). Zinc is also a constituent of crystalline (but not of amorphous) insulin. Whether zinc plays any significant role in connection with the action of insulin or carbonic anhydrase is not known, nor has any specific function been ascribed to zinc in animal nutrition. However, zinc is said to enhance the action of the follicle-stimulating hormone (FSH) and of the luteinizing hormone (LH) of the anterior pituitary.

Trace Elements. Various other elements found in traces in biological material are of uncertain significance. Aluminum has a wide distribution in animals and plants but is not known to be essential, nor does any aluminum added to food during cooking in aluminum utensils have any demonstrable effect on nutrition. However, it has been demonstrated[10] that female albino rats fed biscuits made with a tartrate baking powder gave evidence of a distinct nutritional advantage over rats from the same litter which were fed biscuits made with an alum baking powder. This advantage was apparent from the standpoint of reproduction as well as from that of growth.

Boron is essential for plants but apparently has no significance in animal nutrition. The position of arsenic as a trace element is debatable. Silicon is found in plant and animal tissues but its function is unknown. Bromine has no normal or pathological significance aside from its known pharmacological effects. Selenium may be taken up by plants and thus become transferred to animals where it has a toxic effect which is counteracted by methionine. Molybdenum is an essential component of the enzyme xanthine oxidase.

Acid-forming and Base-forming Foods. Certain foods, such as vegetables and fruits, on burning outside or inside the body leave an ash or residue in which the basic elements (sodium, potassium, calcium, and magnesium) predominate; whereas cereals, meat, and fish foods leave an ash in which the acid-forming elements (chlorine, phosphorus, and sulfur) predominate. Such foods are spoken of as base-forming and acid-forming foods, respectively, and will influence the acid-base balance of the body and the acidity of the urine. Sulfur, while present in foods chiefly in neutral form in the sulfurized amino acids, is oxidized in the body to yield sulfuric acid and is hence an acid-forming element. High-protein foods are hence generally acid-forming. On the other hand, the citrus fruits contain citric acid and acid potassium citrate, the citrate radicals of which are completely metabolized in the body like carbohydrate, leaving behind potassium, which is one of the bases of the body (see p. 1084). Hence many "acid" fruits are base-forming. Grape juice is much less effective than orange juice in reducing urine acidity because the tartaric acid it contains is not completely oxidized but is eliminated to a certain extent in the urine as such. Prunes, plums, and cranberries contain quinic acid. This is excreted in the urine chiefly in the form of hippuric acid (a conjugate of benzoic acid and glycine) which increases the acidity of the urine. With the exception of foods containing incompletely oxidizable organic acids, the acid-forming or base-forming value of foods may be calculated by obtaining the differences between the equivalents of normal acid, calculated from the content of sulfur, chlorine, and phosphorus (considering phosphoric acid as divalent) and of normal alkali calculated from the content of sodium, potassium, calcium, and magnesium. A table of the acid- or base-forming value of various foods will be found in Appendix IV.

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[10] Hawk, *et al.:* Unpublished. For abstract see Philip B. Hawk and Collaborators: *Researches and Writings.* Published privately, 1942.

Through the use of considerable amounts of potatoes or other vegetables or of fruits such as oranges, it is possible to lower the acidity of the urine markedly or even to make it alkaline. Naturally reduction in the amount of acid-forming foods has a similar tendency. Increase of urinary acidity above the usual levels is less readily brought about. Reduction in the acidity of the urine by increasing the solubility of uric acid therein may reduce a tendency to formation of uric acid calculi in the urinary bladder. A certain balance between acid-forming and base-forming foods may be otherwise desirable, since too much acid-forming food might under certain circumstances be a drain upon the fixed base of the body. Fortunately, however, the body has ordinarily a marked ability to protect itself from excess acid formation, through ammonia production and in other ways. It is not clear, therefore, that a harmful effect is produced by a preponderance of acid-forming foods provided mineral, vitamin, and other dietary requirements are met.

EXPERIMENTS ON INORGANIC METABOLISM

1. *Salt-free Diet.* The effect of a salt-deficient diet upon the metabolic processes is reflected in the composition of the urine as shown by the following experiment.

Procedure. **Ingest an ordinary mixed diet containing an ample salt content for a period of two days. Follow this period by the ingestion of a diet which has had its salt content reduced to a very low value. Sugar and olive oil or nonsalted butter may supply the bulk of the calorific part of the diet and dialyzed egg white or casein or commercial protein preparations, e.g., plasmon, gluten, or glidine may supply the protein. Ingest such a diet for three days. (This is an acid-forming diet, p. 1097.) Collect the urine and analyze for chloride, titratable acidity, ammonia, and total nitrogen. Compare the data from the normal days with those obtained when the salt-free diet was ingested.**

2. *Salt-rich Diet.* On an ordinary mixed diet a normal adult will daily excrete 10–15 g. of chloride, expressed as sodium chloride, in the urine. On a salt-free diet this excretion decreases, whereas if the diet contains an excessive quantity of sodium chloride this excess will be promptly excreted in the urine. Normal feces contain very little sodium chloride even after excessive sodium chloride ingestion (see Exp. 3, below).

Experiment. **Ingest an ordinary mixed diet for two days. On each of the following two days take a similar diet plus a weighed amount (e.g., 10 g.) of sodium chloride in divided doses. Collect the urine for the four days in 24-hr. samples, preserve and analyze for sodium chloride (for methods see p. 955). What proportion of the added chloride was recovered?**

3. *Inorganic Elements in the Feces.* The salts of sodium and potassium are almost completely absorbed from the intestine. Hence the alkali metals and chlorides are excreted mainly in the urine and are found only in very small amounts in the feces even when large amounts are ingested. With calcium, magnesium, iron, and phosphate, conditions are different. Ordinarily about 90 per cent of ingested calcium and over half of the magnesium are eliminated by way of the feces. From 20 to 30 per cent of the phosphorus ingested is usually found in the feces.

***Experiments.* (*a*) Ingest for a period of three days an ordinary mixed diet without added salt and containing no milk. Separate the feces for the period (see p. 1046) and retain a portion of the well-mixed feces for analysis.**

(*b*) Proceed as above with the exception that there is added to the mixed diet 10 g. of common salt and a quart of milk (containing about 1.1 g. Ca, 0.1 g. Mg, 1.4 g. chloride expressed as sodium chloride, and 1.0 g. P). Mix the feces well and reserve part for analysis.

Ash 10 g. samples of the feces from the above diets. Dissolve with the aid of a little dilute nitric acid, filter, and make up to 100 ml. Determine in aliquot portions of this solution: (1) Chlorides. (2) Calcium and magnesium. (3) Phosphorus. (For details of analytical methods see Chapter 31.) Calculate the percentages of the added Ca, Mg, P, and Cl which are recovered from the feces.

For a more detailed study of chloride excretion combine this experiment and Exp. 2, above.

4. "Alkaline Tide." For a time after a meal the normal acid reaction of the urine may be changed to neutral or alkaline. This has been explained as due to the production of a temporary alkalosis because of the secretion of the acid gastric juice. The presence of an alkaline tide has been used as an indication of the secretion of hydrochloric acid in the stomach in cases where it was desirable to avoid passing the stomach tube. The urinary ammonia excretion may also serve a similar purpose.

***Experiment.* Ingest an ordinary mixed diet. Urinate just before dinner and note the reaction of the urine to litmus. If acid, determine the hydrogen-ion concentration by the method given on p. 871. (If alkaline, discard the urine and make the test on another day.) After eating a heavy dinner (meats) collect the urine at intervals of half an hour and take the reaction to litmus and determine the hydrogen-ion concentration as before. Did your urine change in reaction after the meal and if so how long a period elapsed between the meal and the occurrence of the maximum change in reaction?**

5. Hydrogen-ion Concentration of the Urine as Influenced by the Ingestion of Acid-forming and Base-forming Foods. It has been demonstrated that vegetables and fruits, on burning, leave an ash in which the basic elements (sodium, potassium, calcium, and magnesium) predominate, whereas cereals, meats, and fish foods leave an ash in which the acid-forming elements (chlorine, sulfur, and phosphorus) predominate.

The acid- or base-forming potentialities of various foods are given in the Appendix. Potatoes, oranges, raisins, apples, bananas, and cantaloupes are important base-forming foods. Among the most important acid-forming foods are found rice, whole-wheat bread, oatmeal, meats, and eggs. Certain fruits, e.g., cranberries, prunes, and plums, yield a basic ash but are acid-forming foods. For further discussion see p. 1097.

On a mixed diet the hydrogen-ion concentration of the urine has been found to average about pH 6.0. Base-forming foods decrease the acidity and the ammonia content of the urine, while acid-forming foods have the reverse effect.

***Experiment.* Ingest a uniform diet consisting of milk, crackers, butter, peanut butter, and water in desired quantities for a period of three days. Follow this by a period of six days during the first three of which considerable quantities of acid-forming foods (see Appendix) are added to the diet. During the**

second half of the period (days four to six) add an abundance of base-forming foods to the diet. Distilled water should be used for drinking purposes and a uniform volume should be ingested daily. Collect the urine in 24-hour periods, preserve, and analyze for hydrogen-ion concentration, titratable acidity, and ammonia (for methods see Chapter 31). Compare your results with those tabulated in the table below.

REACTION OF URINE AS INFLUENCED BY DIET[11]

Determination	*Basal Diet*[12]		*1*	*2*	*3*	*4*	*5*	*6*
			Basal Diet No. 1 Plus				*Basal Diet No. 2 Plus*	
	No. 1	No. 2	Baked potatoes (750 g. per day) (6 days)	Rice (210 g. per day) (4 days)	Cranberry sauce (300–600 g. per day) (6 days)	Bread[13] (whole wheat) 450 g. (1 day)	Prunes (330–550 g. per day) (3 days)	Cantaloup (260 g. per day) (5 days)
	5 days	5 days						
pH	7.19	5.57	7.74	$\underbrace{7.48\text{–}6.90}_{7.14}$	$\underbrace{6.30\text{–}5.70}_{6.19}$	6.80 (Previous day 6.90)	$\underbrace{5.30\text{–}4.80}_{5.07}$	$\underbrace{5.30\text{–}7.38}_{6.70}$
Titratable acidity (ml. 0.1 N)	275	474	$\underbrace{196\text{–}216}_{203}$	$\underbrace{166\text{–}297}_{233}$	$\underbrace{391\text{–}488}_{407}$	350 (Previous day 297)	$\underbrace{570\text{–}540\text{–}578}_{563}$	$\underbrace{466\text{–}250}_{328}$
Ammonia N (grams)	0.310	0.464	$\underbrace{0.221\text{–}0.248}_{0.238}$	$\underbrace{0.166\text{–}0.251}_{0.198}$	$\underbrace{0.219\text{–}0.391}_{0.305}$	0.280 (Previous day 0.251)	$\underbrace{0.602\text{–}0.729}_{0.654}$	$\underbrace{0.513\text{–}0.220}_{0.31}$

6. Hydrogen-ion Concentration of the Urine as Influenced by Alkali Ingestion. The ingestion of certain organic salts of the alkalies, e.g., sodium citrate and sodium bicarbonate, causes an increase in the pH of the urine. The ingestion of acids (either organic or inorganic) or acid salts, e.g. sodium dihydrogen phosphate, decreases the pH of the urine. The alkalies are much more effective in producing changes in reaction than are the acids. The influence of ingested alkali (sodium bicarbonate) is shown in the table on p. 1101 containing data submitted by Henderson and Palmer.

Blatherwick reported a decrease in ammonia nitrogen output from 0.256 g. to 0.072 g., and accompanying decreased acidity under the influence of bicarbonate ingestion (25 g. in two days).

Experiments: Influence of Alkali. **Ingest a uniform diet consisting of milk, crackers, butter, peanut butter, and distilled water for a period of two days. During the next two days take the same diet and ingest 24 g. of sodium bicarbonate between meals (12 in A.M. and 12 in P.M.). Collect the urine in 24-hr.**

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[11] Tabulated from data reported by Blatherwick (*Arch. Int. Med.*, **14**, 409 (1914)). Experiments all made on the same subject (B).

[12] Basal diet No. 1 contained 100 g. graham crackers, 25 g. butter, 400 ml. whole milk ingested at each of the three daily meals. One apple and one soft-boiled egg added at supper. In diet No. 2 whole-wheat crackers were substituted for the graham crackers.

[13] This day was preceded by $NaHCO_3$ ingestion for three days and by rice ingestion for four days.

[14] This diet followed immediately after the diet of prunes (see 5).

INFLUENCE OF INGESTED SODIUM BICARBONATE ON HYDROGEN-ION CONCENTRATION OF URINE

Experiment Number	*Sodium Bicarbonate, Grams*	*pH before Bicarbonate Ingestion*	*Time of Collection of Specimen of Urine and pH*				
			11.00 A.M.	12.00 Noon	1.00 P.M.	2.00 P.M.	3.00 P.M.
1	4	7.40	8.30	7.48	7.48	7.40	5.85
2	8	5.40	8.50	8.30	6.50	6.50	7.40
3	12	5.30	8.70	8.70	8.70	8.70	8.70
4	8	7.40	8.50	8.70	8.50	8.50	8.50
5	8	5.85	..	..	8.70	8.70	8.30
6	8	6.70	7.48	8.70	8.50	8.70	8.50

periods and analyze it for titratable acidity, hydrogen-ion concentration, and ammonia. Compare your results with those shown in table above.

If desired, the bicarbonate may be given in one dose of 8 to 12 g. and the urine collected in hourly specimens for the next 5 hours and each specimen analyzed. Data from such experiments are shown in table above.

7. Influence of Calcium Deficiency. A demonstration of the harmful effect following the elimination of calcium from the diet may readily be made if the diets listed below be fed to young white rats.

Procedure. **Place two young white rats (40–60 g.) in separate cages and feed the diets given in the following table. Make frequent body-weight determinations. The rat receiving Diet 2 will show normal growth. The rat receiving Diet 1 will fail to show normal gains in weight. This diet is deficient in calcium. See Figs. 269 and 270.**

	Diet 1	*Diet 2*
	Per Cent	*Per Cent*
Beef liver (steamed and dried)	20.0	20.0
Casein	10.0	10.0
NaCl	1.0	1.0
KCl	1.0	1.0
$CaCO_3$	0.0	1.5
Dextrin or starch	65.0	63.5
Butter fat	3.0	3.0

8. Influence of Ultraviolet Radiation on Inorganic Metabolism. Ultraviolet radiation has calcium-depositing properties. See p. 1248.

Procedure. **Place three young white rats (litter mates) weighing 50–60 g., in individual cages, and supply ad lib. a rachitogenic diet such as described in Chapter 35. Keep the rats in a dark or dimly lit room, but subject one to an hour's exposure to direct sunlight at about noon each day, and expose another to 1 minute's irradiation at a distance of 2 feet from an ultraviolet lamp. After 4 weeks examine the leg bones roentgenographically or by the line test (see p. 1265), and perform bone ash analyses on the dried, fat-free bones.**

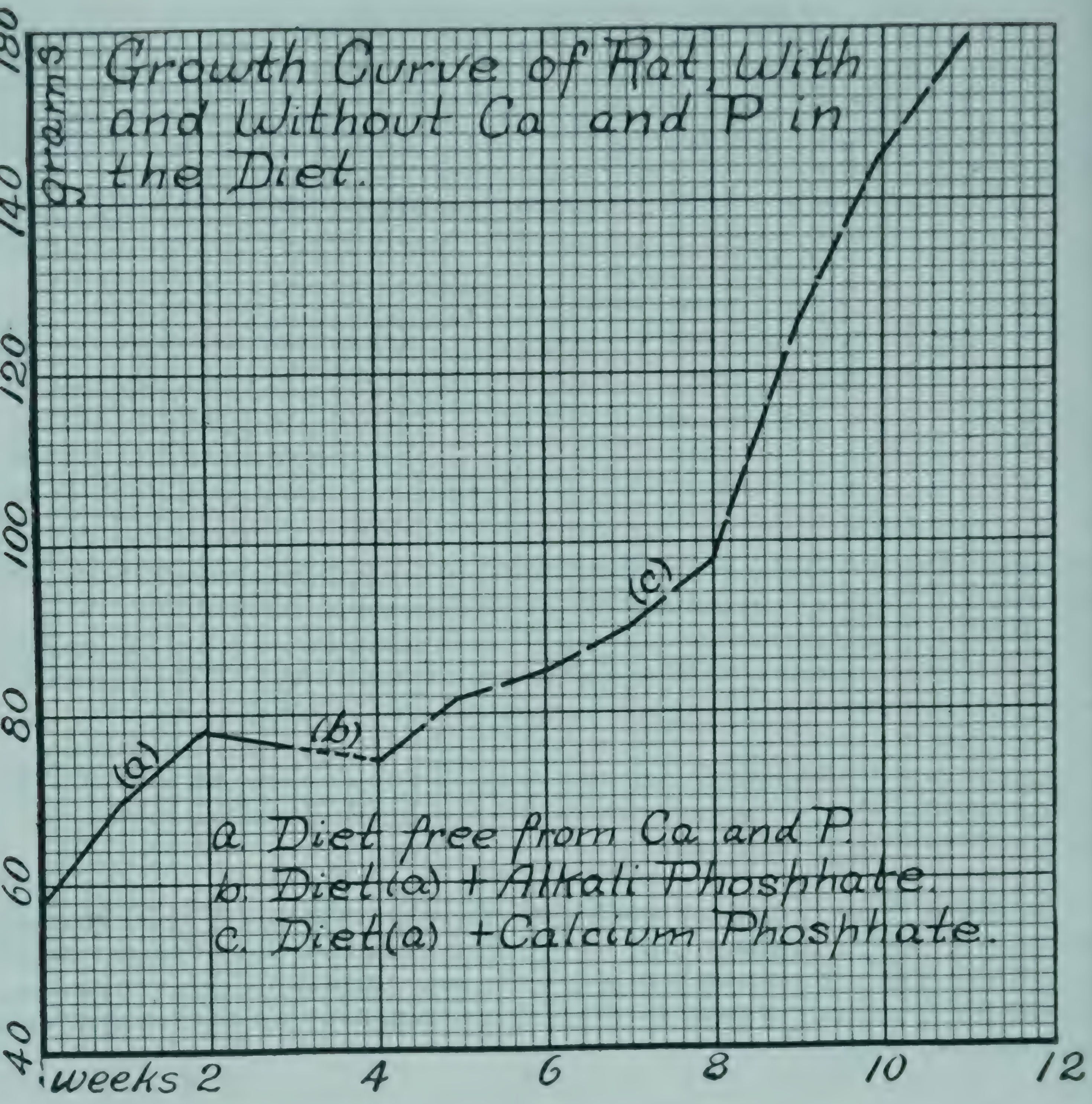

FIG. 269. GROWTH CURVE OF RAT WITH AND WITHOUT CALCIUM AND PHOSPHORUS IN THE DIET.

Unpublished data from the senior author's laboratory.

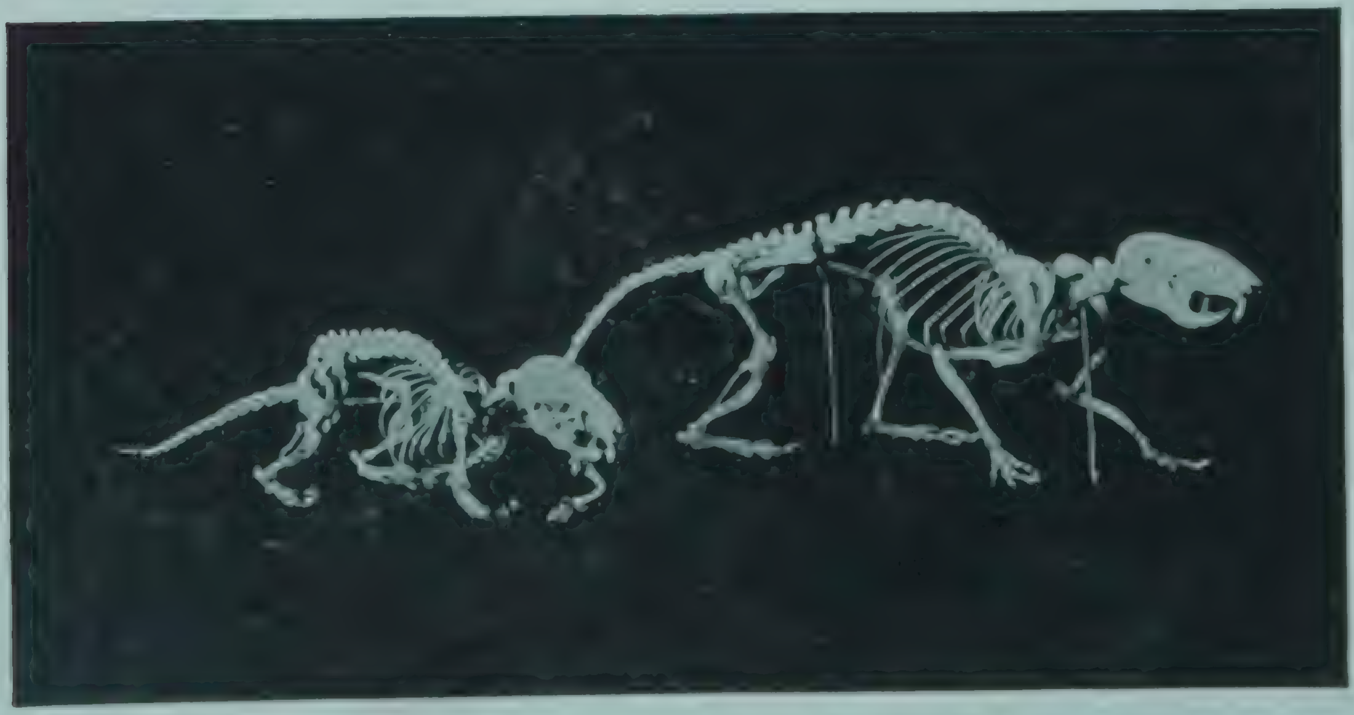

FIG. 270. SKELETONS OF TWIN ALBINO RATS SHOWING INFLUENCE OF CALCIUM DEFICIENCY.

Courtesy, Sherman and MacLeod: *J. Biol. Chem.*, 64, 429 (1925).

9. Influence of Iron and Copper Deficiency.[15] The anemia caused by the ingestion of milk as the sole article of diet may be cured by the addition of an iron (and copper) supplement.

Procedure. **Place three young white rats (litter mates) on a milk diet and perform hemoglobin determinations weekly on blood obtained by clipping off the tips of the tails. After the hemoglobin has dropped to half its original value, feed one rat a daily supplement of 0.25 mg. of Fe (as ferric chloride) and another rat 0.25 mg. of Fe (as ferric chloride) and 0.05 mg. of Cu (as copper sulfate). Continue the third animal on the original milk diet without Fe or Cu supplement. Continue weekly determinations and compare the condition of the three animals, noting especially the color of the tails, ears, and eyes.**

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[15] For quantitative technique for the study of nutritional anemia see Elvehjem and Kemmerer: *J. Biol. Chem.*, **93**, 189 (1931); Smith and Otis: *J. Nutrition*, **13**, 573 (1937).

35

Vitamins and Deficiency Diseases[1]

In 1881 Lunin, a pupil of Bunge, recognized that a diet consisting only of purified protein, carbohydrate, fat, and minerals would not sustain life. When such diets were supplemented with certain natural foods, notably milk, normal growth and well-being of the experimental animals were restored. These findings were in accord with earlier demonstrations of the curative value of citrus fruit juices in scurvy, a disease contracted by sailors, soldiers, prisoners, and others who subsisted on limited fare. They also coincided with observations by the Dutch physician Eijkmann in 1897 that beriberi or polyneuritis, a disease resulting from the prolonged consumption of polished rice, could be cured by supplementing the diet with rice polishings or extracts thereof. These and similar studies suggesting the presence of indispensable substances in natural foods were brought to a head by Hopkins (1912) who called them "accessory food factors." The work of Osborne and Mendel and of McCollum (1913) established beyond doubt the presence of these factors in milk. Funk (1912) introduced the term *vitamine* in the belief that he had isolated one of these factors and established that it possessed the properties of an amine. The final *e* has since been dropped inasmuch as these substances as a class are not related to amines.

A vitamin is a potent organic compound, occurring in minute proportions in natural foods, which must be available to the animal organism from the diet or other sources in order that a specific metabolic process or reaction may proceed normally. The "other sources" referred to in this definition are, for example, ultraviolet irradiation of precursors of vitamin D in the skin or bacterial synthesis of certain vitamins of the B group in the intestinal tract. Unlike amino acids, vitamins do not enter into the tissue structure. Vitamins are exogenous, hormones endogenous. Compounds which may be vitamins for some species may be synthesized by others and hence behave, in effect, as hormones.

With the rapid advancement of our knowledge of the chemical constitution of the vitamins, it is not surprising that structurally related chemical derivatives, both natural and synthetic, should be discovered which exhibit typical physiological activity, though perhaps in varying degree. For these, the term *vitamers* has been suggested. Naturally occurring vitamin D exists in various chemical forms; and what was previously regarded as vitamin A in plant sources is now known to consist of various carotenoids, principally β-carotene, which function as precursors of the vitamin. Hence a distinction should be made between *vitamin activity*, which indicates certain specific biological properties, and the *vitamins per se*, which refer to definite chemical entities, of which there may be several exhibiting similar biological activity.

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[1] The rapid strides being made in this field require that special attention be paid to the date of publication of material on this subject. Important books and review articles on the general subject of vitamins are cited in the Bibliography at the end of this chapter.

Some vitamins are characterized by species specificity; i.e., they are required by certain species of animals and not by others. For example, vitamin C is necessary in the diet of man, guinea pigs, and monkeys, but dogs, rats, and other species are able to synthesize it. Hence for these species vitamin C may be regarded as a hormone. To a certain extent the physiological requirement of various species of animals for vitamins may be met by bacterial synthesis in the rumen or intestinal tract. This process, however, does not signify that these species do not require the nutrient in question.

The vitamins are recognized biologically by their absence rather than by their presence; that is, the characteristic symptoms of each vitamin deficiency must be produced in order that the effect of the vitamin in question can be demonstrated. In addition to (and probably secondary to) their role in the prevention of specific deficiency symptoms, each of the vitamins participates to a greater or less degree in the promotion of growth. Our knowledge of the physiological *modus operandi* of the known vitamins is not far advanced. It is known, however, that most of the vitamins participate in enzymic reactions of a highly complex nature. Vitamins may be precursors of enzymes, as thiamine is of cocarboxylase or niacin of coenzymes I and II; or they may be substrates, as vitamin A is of rhodopsin.

The condition induced by the absence of a single vitamin from the diet is called an *avitaminosis*, e.g., avitaminosis B_1; if the deficiency is multiple, the term *polyavitaminosis* is applied. Many observations, particularly on the effect of deficiency or dosage of one vitamin on the storage or excretion of another, suggest the need for balanced proportions of certain dietary essentials. Until more is known about the specific mechanism of the action of individual vitamins, however, it will be difficult to establish the significance of such interrelationships.

The present list of vitamins cannot be regarded as complete, but it is probable that from the standpoint of human nutrition all the major vitamins have been identified. The table on p. 1106 summarizes the common names of the vitamins, and their principle *vitamers*—i.e., structurally related compounds possessing qualitatively similar activity. Some question has been raised as to the justification for including choline or inositol among vitamins, since such compounds are present in the foods in more than trace quantities. Nevertheless, they do not form a material part of body tissue, and functionally they conform to the definition of vitamins stated above.

Concurrent with the advances in isolation and identification of the vitamins, chemical and physical analytical procedures have been evolved for determining the content of almost every vitamin. More recently,

SUMMARY OF PRINCIPAL COMPOUNDS WHICH POSSESS VITAMIN ACTIVITY

Vitamin		*Principal Vitamers*	
Common Name	*Principal Synonyms*	*Natural*	*Synthetic*
Vitamin A	Axerophthol Antiophthalmic factor Vitamin A_1	α-, β-, and γ-Carotene Cryptoxanthine Vitamin A esters Vitamin A_2 Neo-vitamin A	Vitamin A acid Vitamin A ketone Vitamin A esters
Thiamine	Vitamin B_1 Aneurin Antineuritic factor Antiberiberi factor	Thiamine pyrophosphate (cocarboxylase) Thiamine orthophosphate	Vitamin B_1 disulfide Analog with methyl group in position 6 instead of 2 in pyrimidine ring
Riboflavin	Vitamin B_2 Vitamin G Lacto-, ovo-, verdo-, or hepatoflavin	Riboflavin mononucleotide Riboflavin dinucleotide	7-Methyl-9-(D-1′-ribityl)-isoalloxazine 6-Methyl-9-(D-1′-ribityl)-isoalloxazine 6-Ethyl-7-methyl-9-(D-1′-ribityl)-isoalloxazine
Niacin	Nicotinic acid P-P factor Antipellagra factor Anti-black-tongue factor	Niacinamide Coenzyme I (DPN) Coenzyme II (TPN) N^1-methylnicotinamide	Coramine Esters of niacin
Vitamin B_6	Pyridoxine Anti-acrodynia factor Adermin	Pyridoxal Pyridoxamine Pyridoxal phosphate	
Pantothenic acid	Chick antidermatitis factor Filtrate factor		Esters
Biotin	Vitamin H Skin factor Anti-egg-white injury factor Bios II	Dethiobiotin	Sulfoxide of biotin Esters Dethiobiotin
Pteroylglutamic acid	Folic acid Anti-anemia factor Vitamin B_c	Fermentation L. casei factor Liver L. casei factor	Pteroic acid

SUMMARY OF PRINCIPAL COMPOUNDS WHICH POSSESS VITAMIN ACTIVITY.—(*Continued*)

Vitamin		*Principal Vitamers*	
Common Name	*Principal Synonyms*	*Natural*	*Synthetic*
p-Aminobenzoic acid	Chromotrichia factor Vitamin B_x Anti-gray-hair factor		
Vitamin B_{12}	Cobalamin Cyanocobalamin	Vitamin B_{12a} ($= B_{12b}$) = Hydroxocobalamin	
Choline	Sinkalin Bilineurine Fagin Amanitin	Methionine + ethanolamine Betaine + ethanolamine	Analog containing phosphorus instead of nitrogen Arsenocholine Methyl-diethyl homolog Triethyl homologs
Inositol	Bios I Mouse anti-alopecia factor Rat antispectacled eye factor	Phytin Soybean cephalin	Methyl inositol Inositol hexa-acetate
Ascorbic acid	Vitamin C Antiscorbutic vitamin	Dehydroascorbic acid	6-Deoxyascorbic acid Isoascorbic acid L-Fucoascorbic acid
Vitamin D	Antirachitic vitamin	Vitamin D_2 (Calciferol) Vitamin D_3	Viosterol (irradiated ergosterol) Irradiated 7-dehydrocholesterol Irradiated 22-dihydroergosterol (D_4) Irradiated 7-dehydrositosterol (D_5)
α-Tocopherol	Vitamin E Antisterility vitamin Fertility vitamin	β- and γ-Tocopherols Esters	Esters Analogs with ethyl substituents in place of methyl Analog with NH_2 in place of OH
Vitamin K	Vitamin K_1 Phylloquinone Antihemorrhagic vitamin Coagulation vitamin	Vitamin K_2	Menadione Menadione sodium bisulfite Esters of the hydroquinone forms

RECOMMENDED DAILY DIETARY ALLOWANCES[2]
REVISED 1953

DESIGNED FOR THE MAINTENANCE OF GOOD NUTRITION OF HEALTHY PERSONS IN THE U.S. WHEN CONSUMED IN VARIED DIETS OF COMMON FOODS. ALLOWANCES ARE CONSIDERED TO APPLY TO PERSONS NORMALLY VIGOROUS AND LIVING IN TEMPERATE CLIMATE

Food and Nutrition Board, National Research Council

	Age	*Weight*	*Height*	*Calories*	*Protein*	*Calcium*	*Iron*	*Vitamin A*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic Acid*	*Vitamin D*
	Years	*kg. (lb.)*	*cm. (in.)*		*g.*	*g.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*	*I.U.*
Men	25	65 (143)	170 (67)	3200[3]	65	0.8	**12**	5000	1.6	1.6	16	75	
	45	"	"	2800	65	0.8	**12**	5000	1.4	1.6	14	75	
	65	"	"	2400	65	0.8	**12**	5000	1.2	1.6	12	75	
Women	25	55 (121)	157 (62)	2300[3]	55	0.8	12	5000	1.2	1.4	12	70	
	45	"	"	2000	55	0.8	12	5000	1.0	1.4	10	70	
	65	"	"	1800	55	0.8	12	5000	1.0	1.4	10	70	
	Pregnant (3rd Trimester)			2750	80	1.5	15	6000	1.5	2.0	15	100	400
	Lactating (850 ml. daily)			3300	100	2.0	15	8000	1.5	2.5	15	150	400
Infants[4]	1/12–3/12	6 (13)	60 (24)	kg. × 120	kg. × 3.5	0.6	6	1500	0.4	0.5	4	30	400
	4/12–9/12	9 (20)	70 (28)	kg. × 110	"	0.8	6	1500	0.5	0.8	5	30	400
	10/12–1	10 (22)	75 (30)	kg. × 100	"	1.0	6	1500	0.5	0.9	5	30	400
Children	1–3	12 (27)	87 (34)	1200	40	1.0	7	2000	0.6	1.0	6	35	400
	4–6	18 (40)	109 (43)	1600	50	1.0	8	2500	0.8	1.2	8	50	400
	7–9	27 (59)	129 (51)	2000	60	1.0	10	3500	1.0	1.5	10	60	400
Boys	10–12	35 (78)	144 (57)	2500	70	1.2	12	4500	1.3	1.8	13	75	400
	13–15	49 (108)	163 (64)	3200	85	1.4	15	5000	1.6	2.1	16	90	400
	16–20	63 (139)	175 (69)	3800	100	1.4	15	5000	1.9	2.5	19	100	400
Girls	10–12	36 (79)	144 (57)	2300	70	1.2	12	4500	1.2	1.8	12	75	400
	13–15	49 (108)	160 (63)	2500	80	1.3	15	5000	1.3	2.0	13	80	400
	16–20	54 (120)	162 (64)	2400	75	1.3	15	5000	1.2	1.9	12	80	400

NUTRIENTS NOT TABULATED (excerpted from the original):

Fat. While a requirement for certain unsaturated fatty acids (the linoleic and arachidonic acids of natural fats) has been amply demonstrated with experimental animals, the human need for these fatty acids is not known. . . . Several factors make it desirable (*a*) that fat be included in the diet to the extent of at least 20 to 25 per cent of the total calories, and (*b*) that the fat intake include essential unsaturated fatty acids to the extent of at least 1 per cent of the total calories. At higher levels of energy expenditure, e.g., for a very active person consuming 4500 calories and for children and adolescent persons, it is desirable that 30 to 35 per cent of the total calories be derived from fat.

Water. An ordinary standard for diverse persons is 1 ml. for each calorie of food. . . . At work or in hot weather, requirements may reach 5 to 13 liters daily. Water should be allowed *ad libitum*, since sensations of thirst usually serve as adequate guides to intake except for infants and sick persons.

Sodium Chloride. Average intake of sodium chloride (salt) for the normal adult is 7 to 15 g. daily. . . . The requirement for sodium chloride is increased by environmental and climatic conditions associated with increased sweating. Under unusual conditions, such as doing heavy work in a hot climate, 10 to 15 g. daily, or even more, may be required with meals and in drinking water. However, after acclimatization to heat the sodium content of sweat is greatly reduced and the allowance for salt can be near to normal.

Phosphorus. Phosphorus allowances should be at least equal to those for calcium in the diets of children and of women during the latter part of pregnancy and during lactation. For other adults the phosphorus allowances should be approximately 1.5 times those for calcium. When the calories are obtained largely from cereals, computation of the total phosphorus intake may be misleading because phytin phosphorus may be poorly utilized unless the supply of vitamin D is adequate.

Copper. The requirement for copper for adults is about 1 to 2 mg. daily. Infants and children require approximately 0.05 mg. for each kg. of body weight. The requirement for copper is approximately one-tenth that for iron.

Iodine. The requirement for iodine is small, probably about 0.002 to 0.004 mg. daily for each kg. of body weight, or a total of 0.15 to 0.30 mg. daily for the adult. . . . Especially important in *adolescence* and *pregnancy*.

Vitamin B_6 Group. Vitamin B_6 is essential for human nutrition. . . . The daily intake should approximate about 2 mg.

Folacin. It seems probable that dietary intakes of the order of less than a mg. per day can be expected to cover any nutritional needs for folic acid activity.

Biotin. Intestinal synthesis of this vitamin is extensive and tends to prevent the development of deficiency. . . . Biotin is needed by the human body and cannot be synthesized metabolically.

Pantothenic Acid. The amount of pantothenic acid consumed daily with 2500 calories of good diet is approximately 10 mg.

Vitamin K. Available evidence warrants increased attention to the vitamin K intake of the mother during the latter part of pregnancy. . . . A daily oral dose of 1 mg. should be satisfactory. . . . A suitable amount for the mother preceding delivery is 1 mg. administered parenterally; this amount will stabilize the prothrombin level of the infant until food is taken. A suitable amount for the infant after birth is 1 mg. for a single dose. In fact it has been shown that a single dose of 10 to 20 μg. is adequate to cover the first 5 days. The daily requirement of the infant is approximately 1 μg.

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[2] In planning practical dietaries, the recommended allowances can be attained with a variety of common foods also providing other nutrient requirements less well known; the allowance levels are considered to cover individual variations among normal persons as they live in the United States subjected to ordinary environmental stresses common thereto. [For a proper understanding of the foundations underlying this Table of Recommended Daily Allowances, reference should be made to the original source.]

[3] These calorie recommendations apply to the degree of activity for the reference man and woman described in the original text. For the urban "white-collar" worker they are probably excessive. In any case, the calorie allowance must be adjusted to the actual needs of the individual as indicated by his weight and height. [Adjustment for environmental temperature should also be made.]

[4] The recommendations for infants pertain to nutrients derived primarily from cow's milk or commercial milk preparations. There should be no question that human milk is the most desirable source of nutrients for infants, although expected intakes may not provide the recommended levels of certain nutrients; e.g., protein calcium, and riboflavin.

microbiological procedures have been developed (particularly for the vitamins of the B group) based on the propagation of various bacteria, yeasts, or molds in special media in which one of these vitamins is the limiting factor.

The conditions employed in the nonbiological assay procedures, especially in the preliminary extraction or hydrolysis of the samples, are of course not necessarily equivalent to the conditions to which such foods may be subjected in the gastrointestinal tract. Therefore vitamin *content* as determined by chemical analysis may not always coincide with vitamin *potency* as measured by feeding tests. Correlation of animal assays of foods with nonbiological assays suggests that the full content of vitamins is usually, but not always, biologically active under the experimental conditions employed. The assessment of vitamins in foods in terms of their physiological availability for man is a field of investigation which needs more intensive exploration.

It is important when using tables of the distribution of vitamins in foods, such as that presented in Appendix III, to bear in mind that most of these data are based on assays for total vitamin content rather than for available vitamin content in the physiological sense. For example, in tables spinach, whose "vitamin A" is due to the activity of carotenes, or butterfat, whose "vitamin A" is due to a mixture of carotenes and preformed vitamin A, are usually presumed to have been evaluated biologically; and whenever possible the interpretation of nonbiological assays is made in the light of existing animal-assay data.

When referring to food-composition tables it must be recognized that uniformity is the exception rather than the rule. Such factors as soil, climate, season, varietal differences, and period of harvesting (which affect plant foods) or composition of the feed, age at slaughter, and duration of lactation (which affect animal foods), as well as storage, transportation, processing, "refining," and culinary losses, account for a several-fold range of variation in the vitamin content of natural foods. Hence the values given in tables must be regarded as indicative of expected orders of magnitude rather than absolute vitamin contents.

Under the provisions of the Food, Drug and Cosmetic Act, the Food and Drug Administration established standards of minimum daily requirement for certain of the major vitamins (and minerals) to serve as a basis for proper labeling of foods intended for special dietary uses. These values are presented as a guide to the interpretation of such labeling but are not to be confused with the table of recommended daily allowances prepared by the Food and Nutrition Board of the National Research Council reproduced in its latest (1953) revision on p. 1108. The latter is intended to furnish "desirable goals toward which to strive in planning diets and food supplies." It provides for variations in requirements due to sex, age, activity, and the physiological demands of pregnancy and lactation. Individual needs may deviate significantly from these allowances owing to differences in body weight, glandular activity, gastrointestinal disturbances, and many other conditioning factors.

The term "major vitamins" is sometimes applied to thiamine, riboflavin, niacin, ascorbic acid, and vitamins A and D, for which fairly reliable knowledge exists as to human requirements; it is generally believed that if diets are devised to supply adequate amounts of these vitamins, sufficient quantities of all other vitamins (somewhat presumptuously called "minor") will be present. This view is questionable and merely reflects current ignorance as to the essentiality and quantitative requirements for such vitamins which, it is hoped, future research will correct.

Whether a given food, as prepared for consumption, is a poor, fair, good, or excellent source of the vitamins or of other dietary essentials must be judged by the size of the usual portion and the frequency with which it is consumed. The Council on Foods of the American Medical Association[5] tentatively adopted the following standards for determining the significance of a source of a dietary essential:

1. In general, when one-tenth of the day's requirement for an average man is furnished in a portion which can be easily eaten in one day, the food may be regarded as a "fair" source.
2. When one-tenth of the day's requirement is contributed by an amount of the food which at the same time furnishes not more than 200 calories, the food may be classed as a "good" source.
3. When one-tenth of the day's requirement is furnished by a food which appears in the diet practically every day, and in which the portion contributing one-tenth of the essential furnishes not more than 100 calories, the food may be classed as "excellent."
4. When a food is not one which can be easily eaten in amounts to furnish one-tenth of the day's requirement, or is one eaten infrequently, or both, and the amount required for one-tenth of the day's allowance of the essential furnishes more than 200 calories, the food is a negligible or poor source.

VITAMIN A

Physiological and Clinical Aspects of Vitamin A. Vitamin A was first recognized by the failure of rats to grow on diets lacking in natural fats and oils. At about the time of cessation of growth the eyes become hemorrhagic, keratinized, and later infected. This condition, known as xerophthalmia, is associated specifically with deficiency of vitamin A, hence the name *antiophthalmic* (or *antixerophthalmic*) vitamin (Fig. 271). Interruption of growth (Fig. 272) and lowered resistance to bacterial infection are secondary results of a specific disturbance in the metabolism of epithelial membranes resulting from vitamin A deficiency. This is deduced from the fact that keratinization is observed in the sublingual and submaxillary glands, and in the respiratory, alimentary, and genitourinary tracts, as well as in the cornea and conjunctiva. Similar involvement of the oral and pharyngeal mucosa is responsible, at least in part, for the diminution of food consumption and consequent loss in weight. The formation of urinary and renal calculi observed after vitamin A depletion is probably also a sequel to the metaplastic changes in the epithelial membranes. Similar alterations in mucosal epithelium are followed by bacterial invasion and consequent infections of the sinuses, middle ear, and other areas. However, vitamin A has no specific or immunologic value in the treatment of the common cold. A form of dryness of the skin (xerosis) and a follicular hyperkeratosis (phrynoderma) are among the

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[5] *J. Am. Med. Assoc.*, **108**, 1890 (1937).

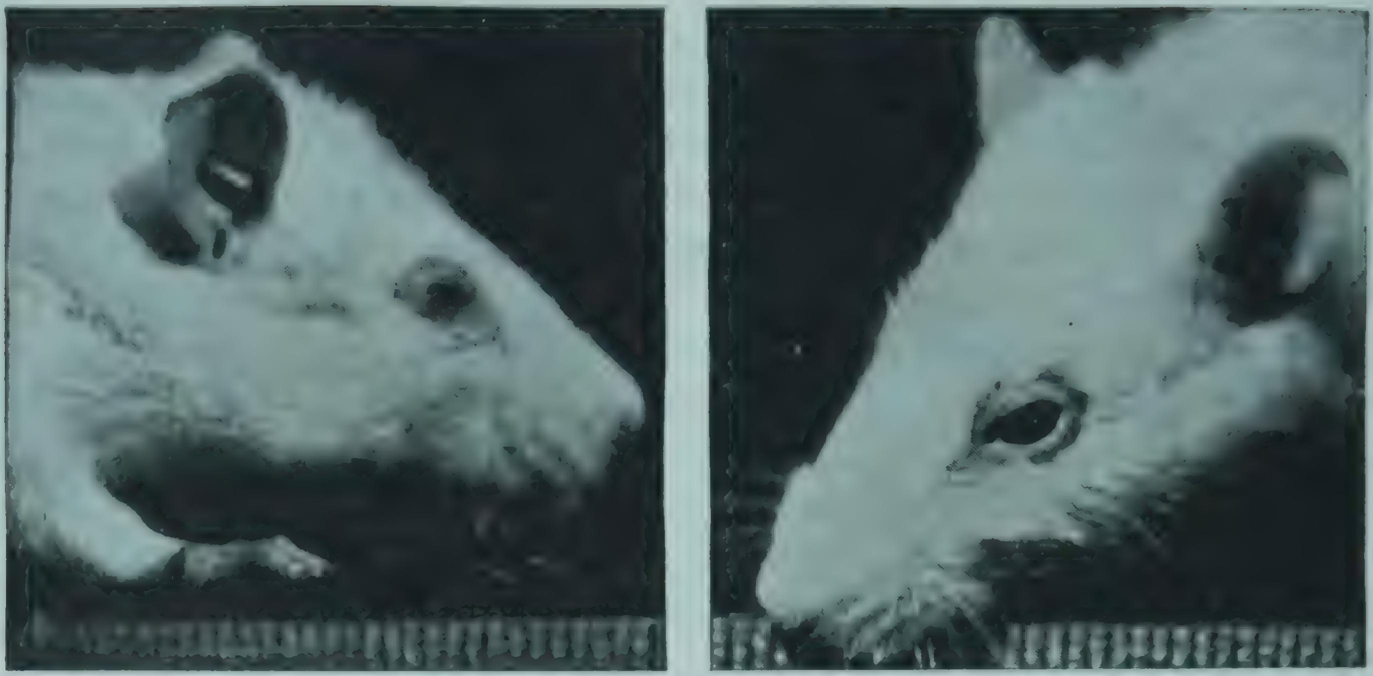


FIG. 271. OPHTHALMIA IN TWO STAGES OF SEVERITY.

(*Left*) Incrustation caused complete closing of eye. (*Right*) Depilated and hemorrhagic area surrounding eye.

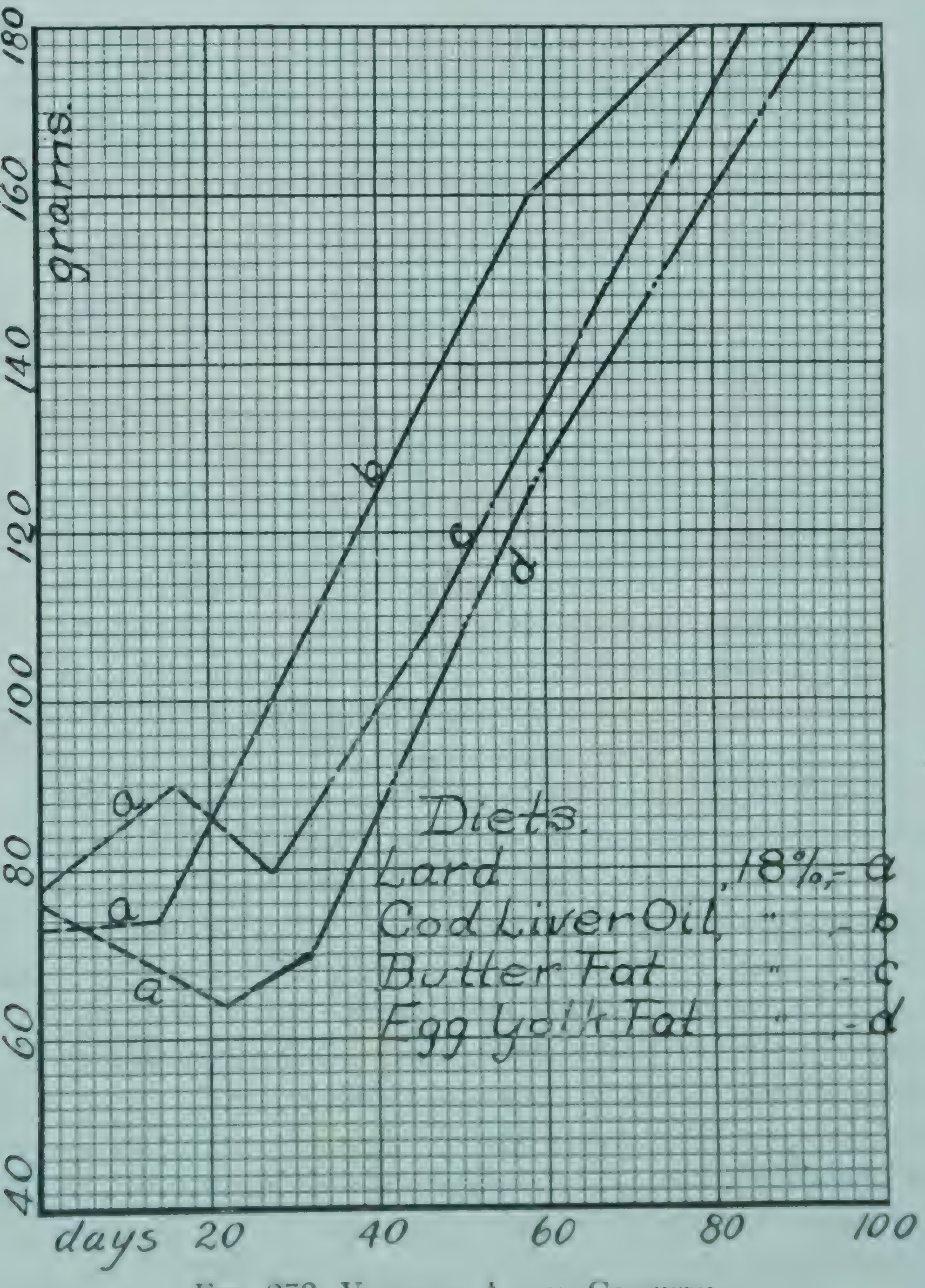


FIG. 272. VITAMIN A AND GROWTH.

The diet containing lard was deficient in vitamin A, while codliver oil, butterfat, and egg-yolk fat are rich in this substance.

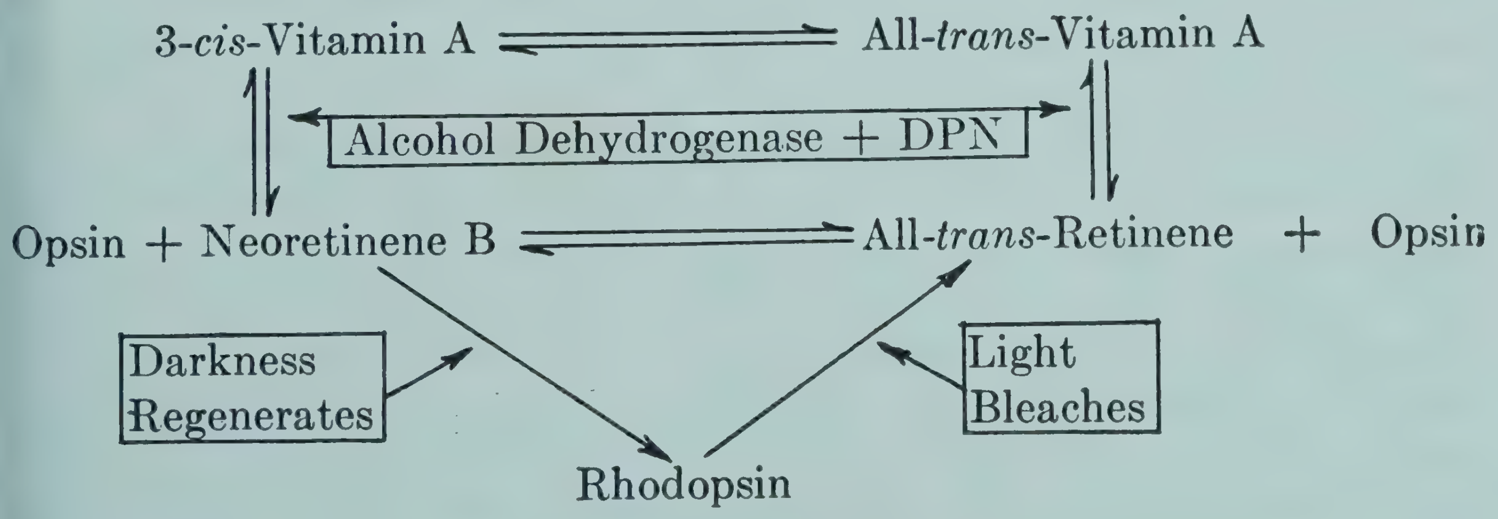
Courtesy, Osborne and Mendel: *J. Biol. Chem.*, 16, 434 (1913); 17, 405 (1914).

symptoms of avitaminosis A found in man as well as in animals. A variety of skin diseases characterized by these symptoms have responded to vitamin A therapy (see p. 1290). Suggestions have been made to name vitamin A "anti-infective"[6] or "antikeratinic,"[7] but these terms have not been adopted because their implications are too broad. Inanition accompanying severe vitamin A depletion may contribute in no small degree to the lowered resistance to bacterial invasion. The primary specific effect of lack of vitamin A, however, is the "substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes and paraocular glands, and the genito-urinary tract" (Wolbach).

There is evidence that lack of vitamin A may produce degenerative changes in the myelin sheath. Since the lack of vitamin A interferes with the process of ovulation, an adequate dietary supply of this vitamin is necessary for normal fertility. It has been demonstrated in rats that a liberal allowance of this vitamin is conducive to longevity.

In addition to its role in maintaining the normal epithelium of the eye and paraocular glands, vitamin A plays a critical part in the process of vision. The characteristic pigments of the retinal rods and cones are conjugated carotenoid proteins known respectively as rhodopsin and iodopsin. According to Wald[8] they differ only in respect to their opsin or protein moieties (called scotopsin and photopsin, respectively). The specific carotenoid common to both is a *cis* isomer of retinene. The range and degree of sensitivity of the rods to light (scotopic vision) is determined by the absorption spectrum of rhodopsin, whereas that of the cones (photopic vision) is determined by the absorption spectrum of iodopsin.

The chemical changes in the visual cycle involving rhodopsin are shown in the following diagram.



In the pigment epithelium of the rods a *cis* isomer of vitamin A alcohol is oxidized to the corresponding vitamin A aldehyde, retinene. This reaction is reversible and is mediated by alcohol dehydrogenase in the presence of cozymase (DPN). The protein opsin, acting as an aldehyde trapping agent, combines stoichiometrically and spontaneously with retinene in the dark to form rhodopsin or visual purple. This is the basis of dark adaptation. Light induces a series of photochemical changes in rhodopsin, beginning with bleaching of the purple pigment on the release of the visual impulse, and ending with the formation of all-*trans*-retinene and its isomerization. For each molecule of retinene formed two sulfhydryl groups are liberated, and in the reverse reaction two free HS groups are provided by opsin.

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[6] Green and Mellanby: *Brit. Med. J.*, **2**, 691 (1928).
[7] Vedder: *Porto Rico J. Pub. Health and Trop. Med.*, **5**, 283 (1930).
[8] Wald: *Harvey Lectures*, **41**, 117 (1945–6); *Science*, **113**, 287 (1951).

The resynthesis of rhodopsin is isomer-specific, requiring a *cis* isomer of retinene (probably 3-*cis* or neoretinene B) for the formation of which the corresponding *cis* isomer of vitamin A is withdrawn from the circulation. Equilibrium reactions involving the isomerization of *trans*-retinene or vitamin A to *cis* forms take place elsewhere in the body, probably in the liver.

The reversible synthesis of rhodopsin has been reproduced *in vitro* by Hubbard and Wald[9] using the four-component system vitamin A (natural or *cis* isomers), cozymase, alcohol dehydrogenase, and opsin.

Since some vitamin A is lost in the visual cycle, deficiency of the vitamin results in a diminution of visual acuity, i.e., the ability to see in subdued light. This condition is known as nyctalopia[10] or night blindness and is the earliest sign of vitamin A deficiency in man, though it may result from other causes. Dark adaptation is the term applied to the adjustment of the visual threshold to darkness after exposure of the eyes to light. Quantitative measurement of the visual threshold—i.e., the light intensity required to elicit a visual sensation—is made by photometric instruments which measure the rate of restoration of visual acuity following stimulation by a light source of standard intensity.[11]

Severe outbreaks of vitamin A deficiency in humans are rare and of limited geographic distribution. The incidence of ophthalmia is relatively high for example in China, Labrador, and India. An acute epidemic among Danish children during World War I was attributed to the substitution of margarine for butter, which was largely exported. The fortification of margarine with vitamin A is now a common practice in most countries where it is sold.

Twenty to 25 units of vitamin A per kg. body weight suffice to maintain a normal visual threshold in most species studied. This amount will also maintain the normal estrus cycle in the rat, although storage in the liver does not occur until approximately four times this level is reached. Liberal allowances of vitamin A, among other factors, tend toward optimum, as distinguished from adequate, nutrition (see p. 1074). In experiments on the rat, Sherman and his associates have shown that by feeding several times the dose of vitamin A sufficient to prevent deficiency symptoms, the reproductive period of females as well as the longevity of both sexes could be prolonged.

The efficiency of absorption of vitamin A in different species is influenced by the nature and quantity of the diluent and by the state of the

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[9] Hubbard and Wald: *Science*, **115**, 60 (1952).

[10] The term "hemeralopia" (literally, day blindness) has been applied improperly to this condition.

[11] Clinical instruments are the Bio-Photometer (manufactured by the Frober-Faybor Company, Cleveland, Ohio) and the Adaptometer of Hecht and Mandelbaum: *J. Am. Med. Assoc.*, **112**, 1910 (1939).

VITAMIN A AND D CONTENT OF OILS FROM FISHERY SOURCES HAVING COMMERCIAL IMPORTANCE IN THE UNITED STATES AND ALASKA*

Fish		*Area*	*Source of Oil*			*Vitamin A, USP Units per g. of Oil*		*Vitamin D, I.U. per g. of Oil*
Common Name	*Scientific Name*		*Organs*	*Per Cent of Body Weight*	*Oil in Organs, Per Cent*	*Range*	*Average*	
Soupfin shark, male	*Galeorhinus zyopterus*	Pacific	Liver	10	55–68	45,000–200,000	120,000	5–25
Soupfin shark, female	*Galeorhinus zyopterus*	Pacific	Liver	10	65–72	15,000–40,000	32,000	5–25
Grayfish (dogfish)	*Squalus suckleyi*	Pacific-Alaska	Liver	10	67–72	2,000–20,000	5,000	5–25
Grayfish (dogfish)	*Squalus suckleyi*	Pacific-U.S.	Liver	10	50–70	8,000–25,000	14,000	5–25
Halibut.......	*Hippoglossus hippoglossus*	Pacific	Liver	1–3	8–27	20,000–160,000	60,000	1000–5000
Halibut.......	*Hippoglossus hippoglossus*	Pacific	Viscera	2.5–5	2–5	70,000–700,000	200,000	100–500
Sablefish......	*Anoplopoma fimbria*	Pacific	Liver	2–2.5	10–26	50,000–190,000	90,000	600–1000
Sablefish......	*Anoplopoma fimbria*	Pacific	Viscera	3–4	5–12	90,000–250,000	125,000	100
Lingcod.......	*Ophiodon elongatus*	Pacific	Liver	1–1.5	8–20	40,000–550,000	175,000	1000–6000
Lingcod.......	*Ophiodon elongatus*	Pacific	Viscera	1.8–3	4–15	10,000–175,000	40,000	100–200
Hammerhead shark	*Sphyrna zygaena*	Pacific-Atlantic	Liver	..	30–40	30,000–120,000	50,000	..
Albacore tuna.	*Germo alalunga*	Pacific	Liver	1.5–2	7–20	10,000–60,000	25,000	25,000–250,000
Bluefin tuna..	*Thunnus thynnus*	Pacific	Liver	..	4–6	25,000–100,000	75,000	20,000–70,000
Bonito........	*Sarda chilensis*	Pacific	Liver	..	4–12	15,000–60,000	35,000	50,000
Swordfish.....	*Xyphias gladius*	Pacific-Atlantic	Liver	1.4–2.6	8–35	20,000–400,000	250,000	2000–25,000
Cod..........	*Gadus callarias*	Atlantic	Liver	3–5	20–60	1000–6000	2000	100–600
Halibut.......	*Hippoglossus hippoglossus*	Atlantic	Liver	1.5–2.5	15–25	..	40,000	1000–5000
Herring.......	*Clupea pallasii*	Pacific	Body	..	5–25	50–300	90	25–160
Menhaden....	*Brevoortia tyrannus*	Atlantic	Body	..	5–20	..	500	..

* Butler: *Commercial Fisheries Rev.*, April 1946, p. 13.

vitamin, i.e., whether carotene, preformed, free, or esterified.[11a] The recommended daily allowances of vitamin A for various sex and age groups are incorporated in the table on p. 1108. These values are based on a consensus obtained under the sponsorship of the National Research Council. An outline of the clinical symptoms of vitamin A deficiency is given in the American Medical Association syllabus on p. 1290.

Storage of Vitamin A. When young animals, previously fed a diet containing vitamin A, are deprived of this vitamin, they continue to grow for a period dependent upon the amount of vitamin A which they have stored. The storage capacity for this vitamin is relatively greater in young animals, i.e., at the age of most rapid growth. The vitamin is stored principally in ester form in the liver and kidneys and to some extent in the lungs. At high levels of intake, rats store more vitamin A in the liver than in the kidneys, but at low intake levels the reverse is true. Liver storage is the basis of a bioassay for vitamin A.

[11a] Week and Sevigne: *J. Nutrition*, **39**, 233, 251 (1949); **40**, 563 (1950).

Distribution of Vitamin A.[12] Preformed vitamin A occurs only in lipides of animal origin, whereas carotenoid precursors occur in the vegetable kingdom, where they are synthesized. The fat of milk may contain both carotenoids and vitamin A in variable proportions, depending on the animal's ration and the extent of conversion of the former into the latter. The chief natural foods of vitamin A value are butterfat (hence milk, cream, cheese, etc.), egg yolk, liver, and pigmented and leafy vegetables (e.g., carrots, tomatoes, pimientos, spinach, lettuce, alfalfa). Vegetable oils as a class are deficient in vitamin A although red palm oil may contain as much as 0.2 per cent of carotenoids. The liver oils of certain species of fish including the shark (notably the soupfin species), swordfish, halibut, mackerel, etc., are extremely high in vitamin A, some oils being several hundred times as rich as codliver oil, one of the earliest known and most abundant sources of both vitamins A and D. There are great variations within species due to storage with age, sex and reproductive phase, marine nutrition, temperature, etc. Natural fish-liver oils are refined or concentrated and blended to standard potencies for therapeutic or food use.

Certain unicellular marine organisms are able to synthesize vitamin A. These provide food for marine plankton, which are consumed by caplin and other small fish, which in turn are consumed by the larger species. The vitamin is also present to a smaller extent in the visceral and body oils of the cod, salmon, and certain other fish.

In the plant world vitamin A activity, being due to carotenoids, is closely associated with pigmentation; the green outer leaves of lettuce are richer than the white center leaves, green tips of asparagus are richer than "bleached" tips, sweet potatoes are richer than white potatoes, yellow corn than white, palm oil (red) than coconut oil. The milk of cows on green pasturage is higher in vitamin A than is the milk of stall-fed animals. No direct quantitative relationship exists between the color of butter or of fish liver oils and vitamin A potency since in animal fats such activity is due principally to the colorless preformed vitamin. (Commercial butter is often artificially colored.) The yellow pigment carotene possesses the physiological activity of vitamin A by virtue of its conversion into the vitamin in the intestinal wall. Hence carotene, or rather certain carotenoids, are provitamins or precursors of vitamin A. In addition to the carotene isomers (see below) other carotenoids which possess vitamin A activity in animals are cryptoxanthin of yellow corn and paprika, myxoxanthin and aphanin of algae.

The synthesis of provitamins A in plants has been shown to be accelerated by light radiations especially of short wavelength, although the ultimate amount of vitamin formed is not increased. There is no evidence that the animal body can produce vitamin A *de novo*, except in the case of the pigeon.[13] Although synthesis of carotenes seems to parallel chlorophyll formation, the latter pigment does not possess vitamin activity.

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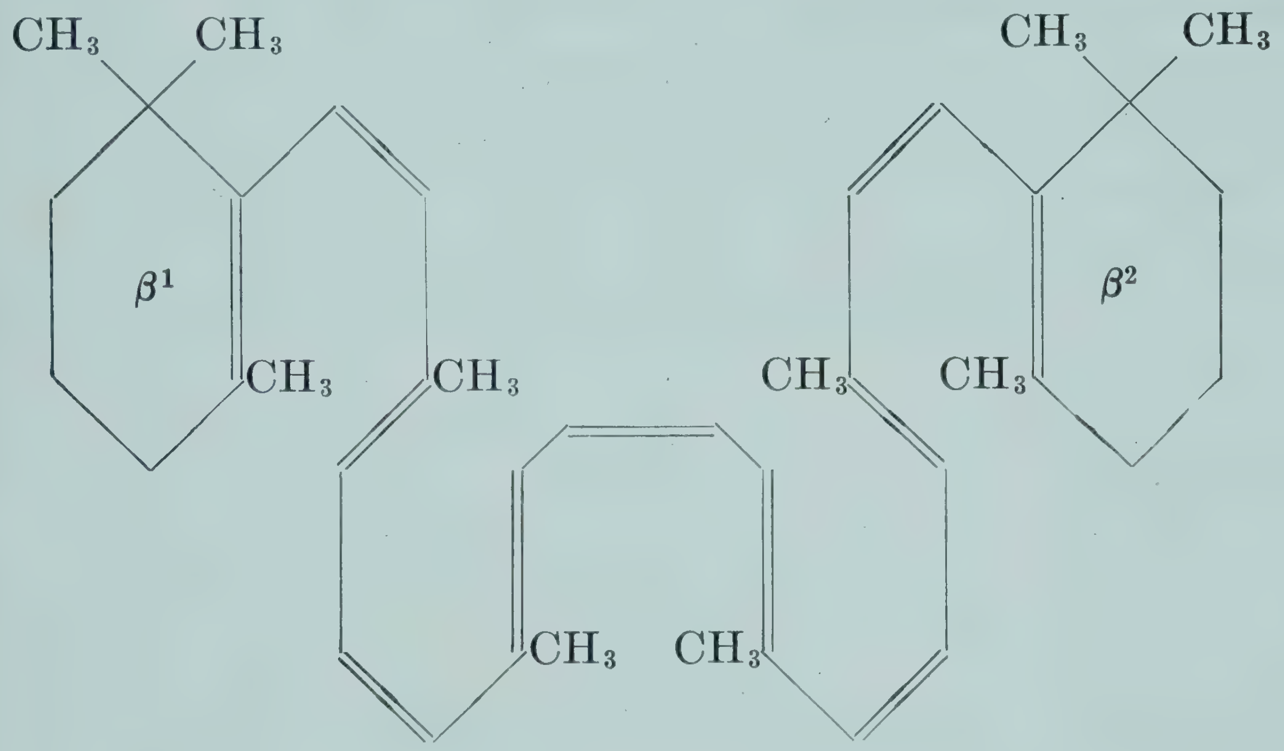
[12] For vitamin A values of foods, see Appendix III.

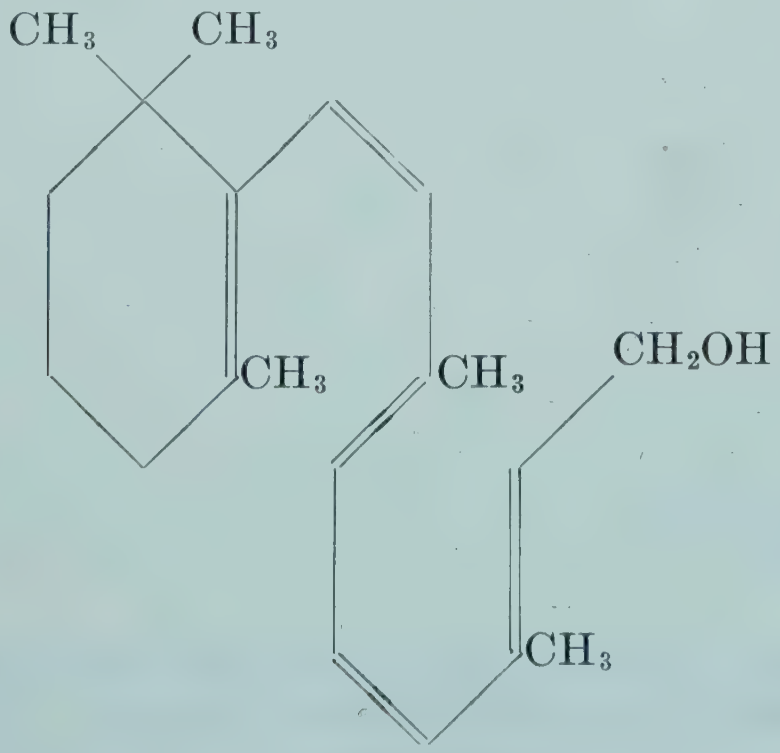
The rate of intestinal absorption of vitamin A and its precursors varies in different species of animals and is influenced by the nature and content of fat in the diet. Excessive quantities of mineral oil inhibit absorption. The efficiency of converting carotenoids into vitamin A likewise varies with the particular compound as well as with the species; in general, rodents are most efficient, pigs and cattle less, and cats least, while the relative capacity of the human infant or adult in this respect remains to be determined.

Chemistry of Vitamin A. β-Carotene has been shown by Kuhn and Karrer to have the following structural formula:



β-Carotene

In the animal organism cleavage occurs at the central double bond with the formation of the alcohol, vitamin A.



Vitamin A

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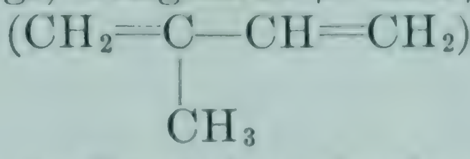
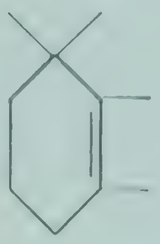
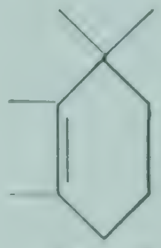
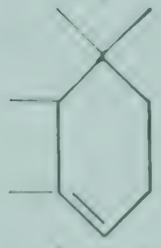
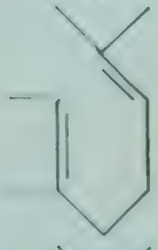
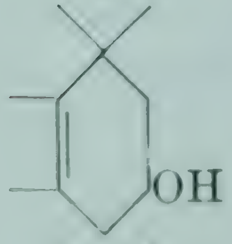
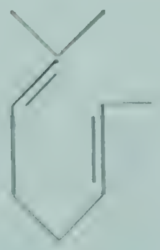
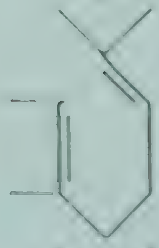
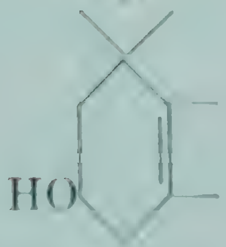
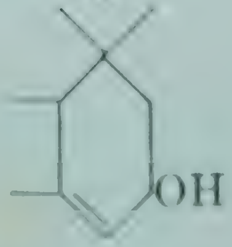
[13] Kon and Drummond: *Biochem. J.*, **21**, 632 (1927).

Hunter and Williams[14] claim to have effected this conversion by oxidation with hydrogen peroxide to give vitamin A aldehyde, and subsequent reduction to the alcohol. In the liver and other storage depots of the animal organism vitamin A exists in the form of fatty acid esters. The palmitate and acetate are produced commercially from either natural or synthetic sources.

It will be noted that the β-carotene molecule is symmetrical, containing two β-ionone rings, designated β^1 and β^2 respectively, bridged by a chain of four isoprene  $(CH_2{=}\underset{\substack{|\\ CH_3}}{C}{-}CH{=}CH_2)$ units. The α and γ isomers differ in respect to the configuration of only one of the rings, the molecular formula of all of these isomers being $C_{40}H_{56}$. In place of the rings indicated above for β-carotene, certain other carotenoids differ only in respect to the rings as shown in the following tabulation.

	β^1 ring	*β^2 ring*
β-Carotene **$C_{40}H_{56}$**		
α-Carotene **$C_{40}H_{56}$**	as above	
γ-Carotene **$C_{40}H_{56}$**	as above	
Cryptoxanthin **$C_{40}H_{55}OH$**	as above	
Lycopene **$C_{40}H_{56}$**		
Xanthophyll (Lutein) **$C_{40}H_{54}(OH)_2$**		

At least one intact β-ionone ring is essential for vitamin A activity, since isomers or derivatives in which the only change is in both rings (e.g., lycopene or xanthophyll) are inactive. The conjugated double bonds in the side chain (or in the carotenoid bridge) make possible a large number of *cis-trans* isomers.[15] However not all of these stereoisomers exist in nature or have been produced synthetically, owing to steric hindrances. Twenty *cis-trans* isomers of β-carotene are possible and even larger numbers in the case of the asymmetrical compounds like α- or γ-carotene. The stereoisomers are differentiated by their spectral-absorption characteristics in both the visible and ultraviolet wavelengths and by their chromatographic adsorption affinities.

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[14] Hunter and Williams: *J. Chem. Soc.*, 554 (1945); Hunter: *Nature*, **158**, 257 (1946).

It has been postulated that because of steric interference, only two of the four double bonds in the side chain of vitamin A assume *cis-trans* configuration, yielding four stereoisomeric forms, viz. all-*trans*-, 3-*cis*-, 5-*cis*-, and 3,5-di-*cis*-vitamin A. The isomers found in liver oils are principally all-*trans* (Fig. 273) associated with a *cis*-isomer (probably 5-*cis*) which has been crystallized[16] and designated "neovitamin A" (Fig. 274). Despite the functional differences between these isomers in the visual cycle (see p. 1113) the growth-promoting activity of neovitamin A for the rat appears to be approximately 80 per cent of that of crystalline all-*trans*-vitamin A.[17]

The approximate vitamin A activity for rats of certain important natural carotenoids relative to β-carotene is as follows:

β-Carotene (all-*trans*)	100
α-Carotene (all-*trans*)	53
γ-Carotene (all-*trans*)	27
Cryptoxanthin (all-*trans*)	57
Neo-β-carotene U(mono-*cis*)	38
Neo-β-carotene B (di-*cis*)	53
Lycopene	0
Xanthophyll	0

β-Carotene is approximately twice as potent biologically as its naturally occurring isomers. It was believed that fission of the carotenoid molecules occurs at the central double bond yielding two molecules of vitamin A; there is evidence, however, that asymmetrical breakdown to the active β-ionone fragment of the molecule can also occur.

Since intestinal absorption, stability, and efficiency of conversion affect the biological activity of carotenoids, the estimation of vitamin A potency of plant foods from the relative content and activity of the individual pigments is not always reliable.

The previous international standard of vitamin A activity, β-carotene, has been replaced by crystalline vitamin A acetate. The International or USP unit is now defined as 0.30 μg. of vitamin A alcohol, equivalent to 0.344 μg. of the acetate. For the estimation of carotene the former standard has been retained; 0.6 μg. of pure β-carotene is considered to have the

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[15] For reviews of stereoisomerism of the carotenoids and vitamin A, see Zechmeister: *Vitamins and Hormones*, **7**, 57 (1949); Karrer and Junker: *Carotenoids*, New York, Elsevier Press Inc., 1950.

[16] Robeson and Baxter: *Nature*, **155**, 300 (1945).

[17] Harris, Ames, and Brinkman: *J. Am. Chem. Soc.*, **73**, 1252 (1951).

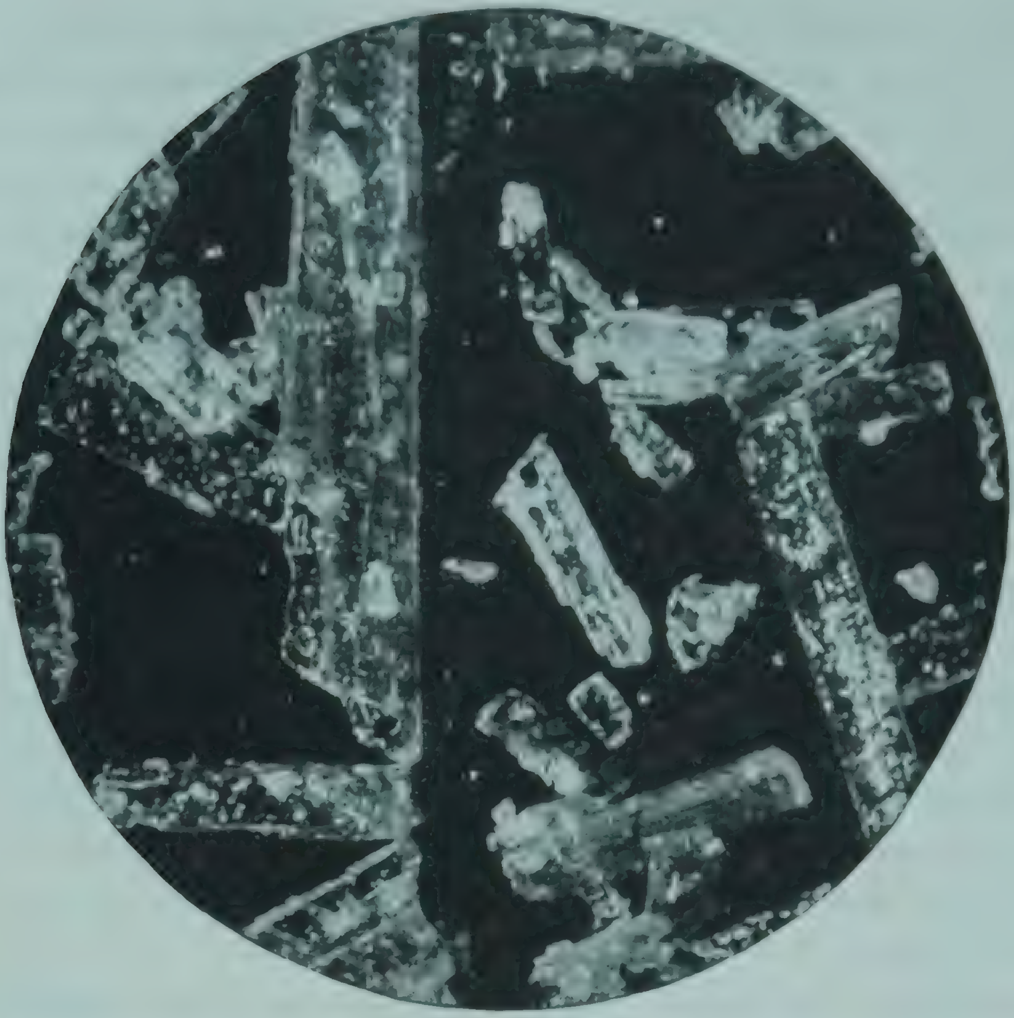

FIG. 273. CRYSTALLINE VITAMIN A ALCOHOL (HIGH MELTING).

Courtesy, Distillation Products, Inc., Rochester, N. Y.

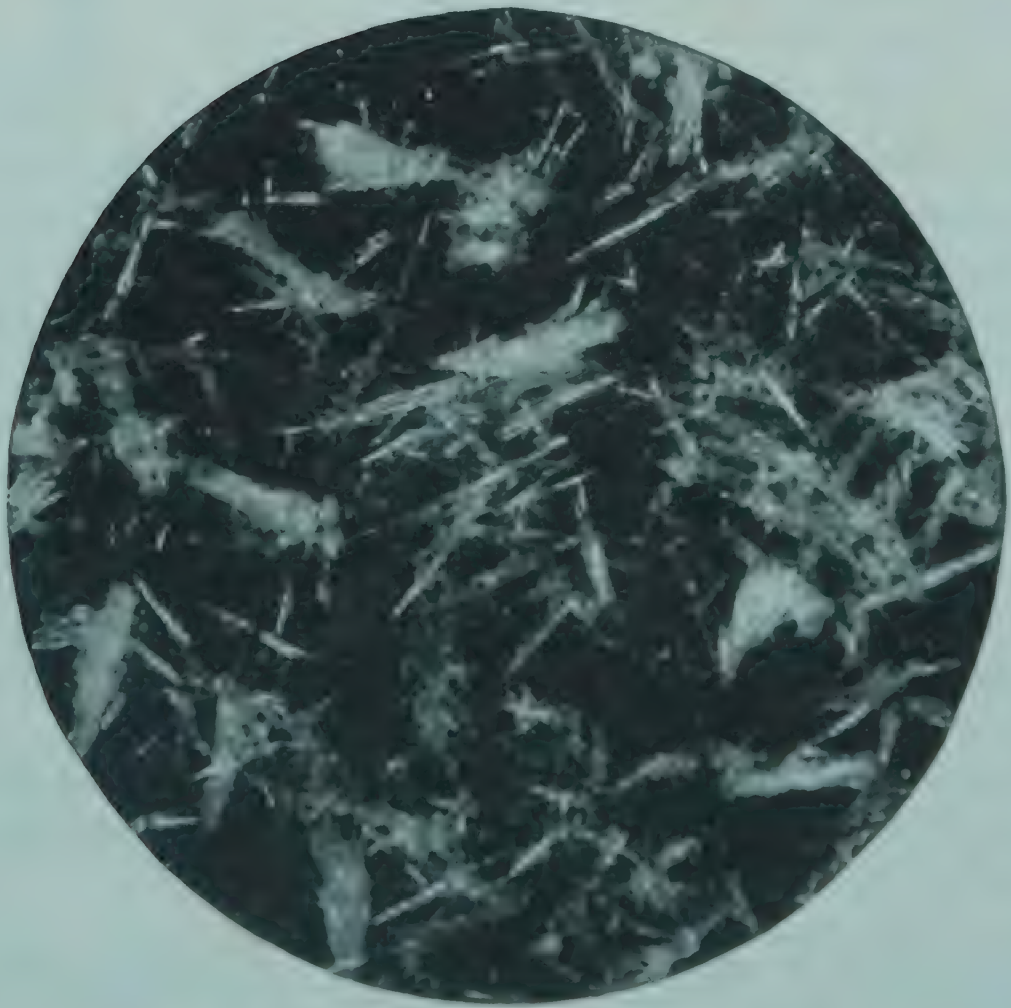

FIG. 274. CRYSTALLINE NEOVITAMIN A.

Courtesy, Distillation Products, Inc., Rochester, N. Y.

activity of one unit of vitamin A. It has been found however that the "crude carotene" of many plant foods (i.e., the pigment in the petroleum ether extract which absorbs light at 420 mμ) has a vitamin A activity of about 1 USP unit per μg.

Vitamin A alcohol forms pale yellow crystals melting at 63–64° C. when deposited from ethyl formate solution or at 7–8° C. when methyl alcohol is the solvent. It forms esters but is not precipitated by digitonin, providing a means of separation from cholesterol which likewise occurs in the nonsaponifiable fraction of animal fats. The vitamin is distillable *in vacuo*, thus forming the basis for a process of concentration from fish liver oils; viz., Hickman's method of molecular distillation. Vitamin A forms colored compounds with certain condensing agents, one of which, antimony trichloride, produces an evanescent blue color (Carr-Price reaction) of sufficient intensity to afford a measure of its concentration. This color absorbs light maximally at 620 mμ, in contrast with the more stable greenish-blue color produced with carotene, which shows an absorption maximum at 590 mμ. For the analysis of foods or animal tissue containing both factors, the $SbCl_3$ reaction necessitates either correction for the differences in rate of color development and fading, or preferably, preliminary separation of the carotenes from preformed vitamin A.

Vitamin A is characterized by an absorption band in the ultraviolet with a maximum at 328 mμ. This forms the basis of the spectrophotometric method of estimating (preformed) vitamin A content.

In the absence of air, vitamin A is quite stable at high temperatures, but when exposed to air or oxygen it is readily destroyed. Aeration of cod-liver oil for 12 hours at 100° C. oxidizes the vitamin A, leaving the antirachitic potency unimpaired. Vitamin A appears to be more resistant to oxidation in its natural environment, probably because of the presence of protective antioxidants such as tocopherols, phospholipides, and possibly other agents. Certain phenolic substances, like hydroquinone and pyrogallol, are even more effective antioxidants, but their use is not permitted on toxicological grounds. Light, especially in the ultraviolet range, exerts a destructive influence upon vitamin A; hence the practice of dispensing fish liver oils in dark bottles and of conducting analytical operations in amber or red glassware.

DETERMINATION OF VITAMIN A

In estimating vitamin A in natural products a distinction must be drawn between vitamin content and vitamin potency. Vitamin A activity is derived principally from the physiologically available amounts of preformed vitamin A, which may be present as free alcohol or as esters, and of the carotenoids, which vary in their individual potencies. Furthermore activity is influenced by the nature and content of fat in the diet and by the physical state of the carrier; for example, fine dispersions of vitamin A in aqueous media are absorbed more rapidly than is the vitamin in oily media.

Because of this distinction between vitamin A content, in the chemical sense, and biological activity of true vitamin A and related compounds, the bioassay continues to play an important function, especially in the evaluation of foods whose vitamin activity is of multiple origin. In products whose vitamin A activity is derived from less complex sources, such as the crystalline alcohol or its esters, high-potency fish liver oils or β-carotene preparations, it is only necessary to establish correlation between values for vitamin content obtained by physical or chemical methods and the bioassay values to justify the use of the nonbiological procedures. Because of the small size of sample available, the analysis of blood and tissues for vitamin A is limited to the nonbiological methods.

CHEMICAL METHODS

Colorimetric Methods for Preformed Vitamin A and Provitamin A. The carotenes are determined colorimetrically preferably after isolation by saponification, extraction, adsorption, and elution. Prediction of biological potency from the content of carotenoids is complicated by their individual differences in activity and absorbability. Chromatographic separation of the carotenoids and their identification and estimation from spectrophotometric absorption data afford a more accurate but involved basis for estimating the vitamin A activity of plant carotenoids.

Various color reactions have been proposed for the estimation of preformed vitamin A, especially in liver oils.[18] The most widely used reaction is that of Carr and Price.[19] When a solution of antimony trichloride in chloroform is added to a dilute solution of a vitamin A-bearing oil, a blue color appears which soon reaches a maximum intensity and then rapidly fades or changes to reddish-brown or other colors, varying with the individual oils. Under carefully controlled conditions the blue color persists long enough to make accurate readings possible. Glycerol 1,3-dichlorohydrin activated by distillation over antimony trichloride is also used as a colorimetric reagent for vitamin A, greater color stability being claimed for the reaction product.[20]

Antimony Trichloride (***Carr-Price***) ***Reaction:*** **Into a dry test tube introduce 0.2 ml. of a 20 per cent solution of codliver oil in chloroform.[21] Add rapidly 2 ml. of a saturated solution of antimony trichloride in chloroform. Observe the color changes.**

Determination of Carotene (***Provitamin A***). β-Carotene occurs in certain plant sources (e.g., carrots, alfalfa) relatively free from other carotenoids such as cryptoxanthin or lycopene. In these cases it may be determined by methods involving selective solvent extraction (phasic separation). Mixtures of hexane and acetone, methanol, or diacetone alcohol are widely used, since they permit partition of the carotenes from xanthophyll, chlorophyll, or other inactive pigments. When present with other carotenoids, β-carotene is preferably separated by chromatography of a hexane solution.

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[18] These have been reviewed by Fisher and Bailey: *J. Assoc. Official Agr. Chem.*, **13**, 352 (1930); Bacharach and Smith: *Analyst*, **59**, 70 (1934), and others.

[19] Carr and Price: *Biochem. J.*, **20**, 497 (1926).

[20] Sobel and Werbin: *J. Biol. Chem.*, **159**, 681 (1945); *Ind. Eng. Chem., Anal. Ed.*, **18**, 570 (1946); *Anal. Chem.*, **19**, 107 (1947). Applications of this reagent to the determination of vitamin A in serum and milk have also been reported. Sobel and Snow: *J. Biol. Chem.*, **171**, 617 (1947); Sobel and Rosenberg: *Anal. Chem.*, **21**, 1540 (1949).

[21] The chloroform should be washed two or three times with its own volume of water, dried over anhydrous Na_2SO_4, decanted and distilled, and the first 10 per cent of the distillate rejected. During these processes the chloroform should be protected from light.

Extraction. **In the case of animal or dairy products containing fat, saponification and extraction is necessary. For this purpose the isolation of the unsaponifiable extract as described for vitamin A on p.1124 may be employed, but the residue after evaporating the ethyl ether should be taken up in an appropriate volume of hexane. Dried plant products or feed mixtures may simply be extracted for one hour under reflux with a mixture of acetone and hexane (3 + 7), using about 30 ml. per 2 g. of sample. After cooling, the extract is filtered through anhydrous sodium sulfate into a 100-ml. volumetric flask and made up to volume with hexane rinsings.**

Other extraction methods have been described for special cases, e.g., those requiring cold extraction to avoid isomerization.[22]

Chromatography. **In view of the predominance of β-carotene in most animal tissues or foods intended for human use, the method to be described, which actually measures total or "crude" carotenes, will generally be found satisfactory. For procedures effecting more complete resolution of the carotenes and their isomers the reader is referred to the literature.**[23]

Insert a chromatographic adsorption tube (22 × 175 mm.) into a suction flask and plug the outlet orifice with a bit of glass wool. Introduce a mixture of equal parts of activated magnesia[24] **and Hyflo Supercel**[25] **to a depth of 15 cm. with the aid of suction and a flat-surfaced tamping device; gently press the adsorbent down to a depth of 10 cm. Cover with a 1-cm. layer of anhydrous sodium sulfate.**

Under continuous suction pour the extract into the adsorption column. (Always keep the top of the column covered with a layer of solvent.) Follow through with 50 ml. of acetone-hexane (1 + 9) to develop the chromatogram. Collect the eluate, which contains all the carotene, leaving the xanthophylls, chlorophyll, and oxidation products in the column. Transfer the partially evaporated eluate to a 100 ml. volumetric flask and make up to volume with acetone-hexane.

Spectrophotometry. **The solution should have an absorbancy corresponding to 1–4 μg. of carotene per ml. Read the optical density in a spectrophotometer at 436 mμ. Convert to "total carotenes" by means of a calibration chart prepared from graded concentrations of a standard solution of crystalline β-carotene;**[26] **or calculate from the following equation:**

$$\frac{(-\log T) \times v \times 100}{196 \times L \times C} = \text{mg. carotene per 100 g. sample}$$

where $(-\log T)$ is the optical density, v the final volume of eluate (100 ml.), L the cell depth in cm., and C the weight of the original sample.

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[22] Beadle and Zscheile: *J. Biol. Chem.*, **144,** 21 (1942); Zechmeister and Polgar: *J. Am. Chem. Soc.*, **64,** 1856 (1942).

[23] Bickoff, *et al.: J. Assoc. Official Agr. Chemists*, **31,** 633 (1948); **32,** 766 (1949). These authors employ hydrated lime as an adsorbent to separate the carotene isomers.

[24] Micron Brand No. 2642, Westvaco Chlorine Products Co., Newark, Col.

[25] Johns-Manville Co., Chicago, Ill.

[26] The International Standard obtainable from USP Reference Standards, 46 Park Ave., N.Y. 16. Pure β-carotene or a mixture (90 per cent β, 10 per cent α) as supplied by General Biochemicals, Inc., Chagrin Falls, Ohio, may be used. β-carotene may be purified as follows: Dissolve 100 mg. β-carotene in 2 ml. chloroform, and reprecipitate with 20 ml. methanol. Filter, wash with a few drops of methanol, and dry in a vacuum desiccator.

Determination of Preformed Vitamin A (Colorimetric Method of Oser, Melnick, and Pader):[27] ***Principle.*** The blue color produced when vitamin A reacts with antimony trichloride in chloroform solution is measured photometrically. An internal standard (increment) is included to compensate for the effect of inhibitors or accelerators on the color development.

Procedure: Preparation of the Unsaponifiable Fraction. **Perform all operations in amber glassware. Into an Erlenmeyer flask transfer a weighed sample, containing preferably at least 50 USP units of vitamin A. The sample may be weighed by difference from a weighing bottle furnished with a rod and stopper. Saponify by refluxing on a boiling water bath for 0.5 hr. with freshly prepared 0.5 N alcoholic potassium hydroxide, using 15 ml. for each gram of sample solids. Cool, and transfer to a separatory funnel, adding an equal volume of water as a wash. Extract four times with 75 ml. of freshly redistilled ether, and discard the aqueous phase. Wash the combined ether extracts once with 50 ml. of water, once with 25 ml. of 0.5 N aqueous potassium hydroxide, and then with 50-ml. portions of water till the last washing gives no color with phenolphthalein. Dry the ether extract with anhydrous sodium sulfate and evaporate to dryness on a water bath, removing the last few milliliters at room temperature with a stream of nitrogen. Dissolve the residue immediately in sufficient purified**[28] **chloroform to produce a concentration of from 5 to 15 USP units of vitamin A per ml. A turbid chloroform solution may be clarified with anhydrous sodium sulfate.**

Colorimetric Procedure. **A direct-reading photoelectric colorimeter with a 620-mμ filter is used for the measurements. Set the instrument at 100 per cent transmittance with a solution containing 1 ml. of chloroform and 9 ml. of antimony trichloride reagent.**[29] **To another tube add 1 ml. of the test solution and 9 ml. of chloroform, and read (*A*). To a tube containing 1 ml. of chloroform extract of the sample, add 9 ml. of antimony trichloride reagent rapidly from a blow-out pipet and measure the maximal blue color as indicated by the full swing of the galvanometer usually attained within 4 seconds (*B*). To another tube containing a 1 ml. aliquot of the test solution, with a micropipet add 0.1 ml. of a vitamin A standard**[30] **containing 10 USP units (or 3 μg.) of vitamin A followed by 9 ml. of the antimony trichloride reagent. Measure the maximal color (*C*).**

CALCULATIONS. Convert galvanometer readings, G, expressed in per cent transmittance, to photometric densities, $P.D.$, as follows:

$$P.D. = 2 - \log G$$

Then,

$$\frac{B - A}{1.01C - B} \times 10 \text{ USP units (or } 3\ \mu\text{g.)} \times \text{dilution factor}$$
$$= \text{USP units (or } \mu\text{g.) of vitamin A per g. of sample.}$$

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[27] Oser, Melnick, and Pader: *Ind. Eng. Chem., Anal. Ed.*, 15, 724 (1943); Oser, Melnick, Pader, Roth, and Oser; *ibid.*, 17, 559 (1945).

[28] Wash reagent grade chloroform three times with an equal volume of water and dry over anhydrous sodium sulfate. Redistil on the day used.

[29] A 25 per cent solution of antimony trichloride in dry chloroform. Filter if solution is turbid.

[30] The USP Reference Standard Solution of crystalline vitamin A acetate may be employed as the standard; the results are expressed in terms of vitamin A alcohol.

Spectrophotometric Method.[31] Since vitamin A is characterized by selective absorption in the ultraviolet, the intensity of absorption at the maximum (Fig. 275) serves as a measure of vitamin A content. Inasmuch as this region of the spectrum is beyond the visible range, spectrophotometric equipment is necessary. Adaptations of this method have been particularly useful in the assay of fish liver oils where the vitamin A activity is due entirely to the vitamin *per se*. Greater accuracy is obtainable by the removal of interfering substances present in the saponifiable fraction, particularly in oils containing less than 15,000 units of vitamin A per gram.

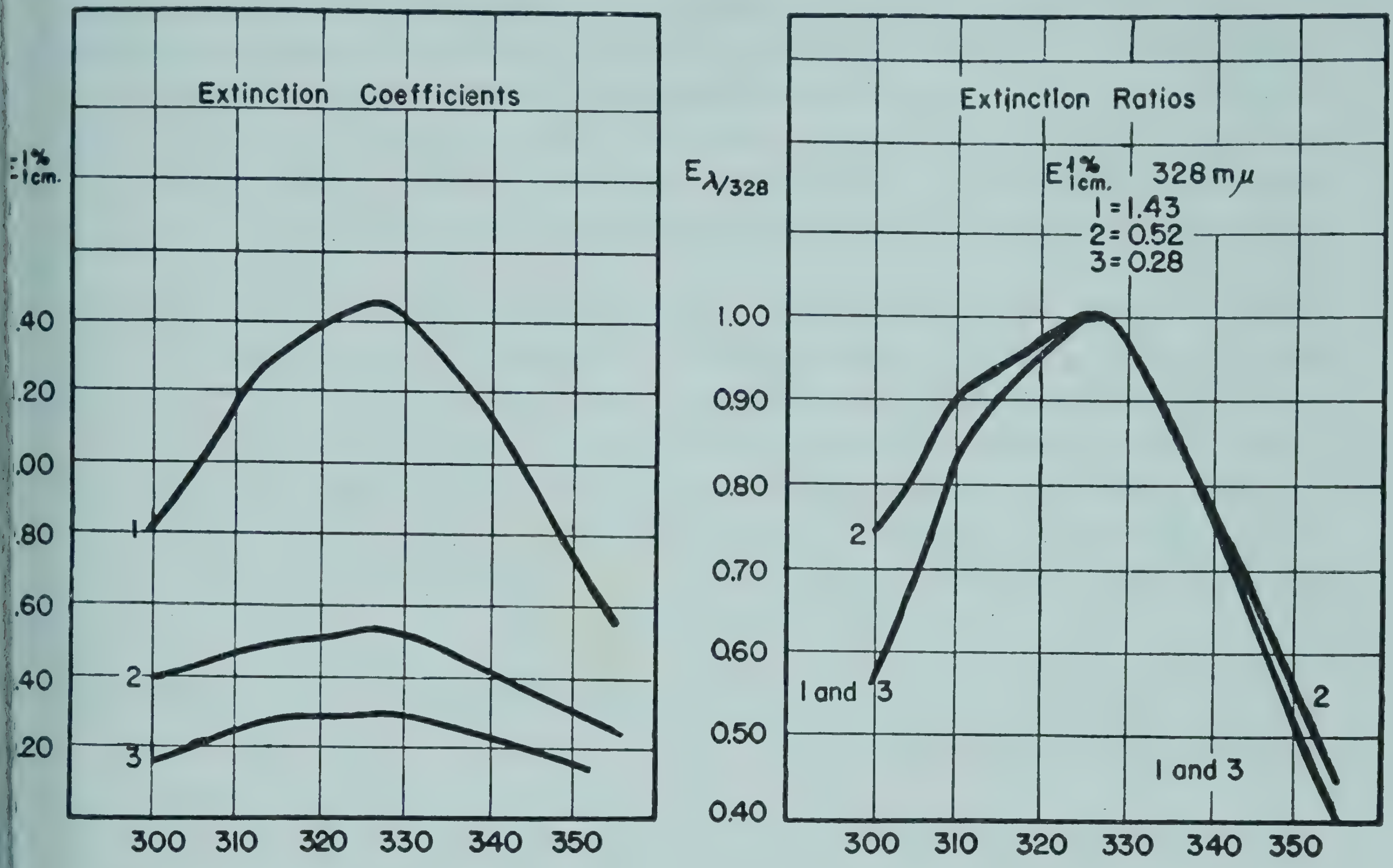

FIG. 275. ADVANTAGE OF PLOTTING EXTINCTION RATIOS RATHER THAN EXTINCTION COEFFICIENTS IN PRESENTING ULTRAVIOLET-ABSORPTION CURVES OF VITAMIN A SOLUTIONS.

1. Vitamin A acetate in ethyl laurate. 2. Oxidation of (1) by air. 3. Dilution of (1) 1:5 with ethyl laurate.

The method involves the comparison of the absorption of ultraviolet radiation through the test solution containing vitamin A, with that absorbed by the solvent alone. Results are usually expressed in terms of the extinction coefficient[32] derived as follows:

$$E^{1\%}_{1\text{cm.}} = \frac{1}{cd} \log_{10} \frac{I}{T}$$

in which $E^{1\%}_{1\text{cm.}}$ is the extinction coefficient, c is the concentration (in per cent), d the thickness of the cell (in cm.), and I and T the intensity of the

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[31] For treatments of this subject, see Chapter 23; also Morton: *The Application of Absorption Spectra to the Study of Vitamins, Hormones and Coenzymes*, 2d ed. London, Hilger, 1942; Chapter 4 in Wokes: *Applied Biochemistry*, Baltimore, Wm. Wood and Co. (The Williams & Wilkins Co.), 1937; and *U.S. Pharmacopeia XIV*, 1950, pp. 756–8.

[32] "Specific absorbancy" in the terminology of modern optical physics.

"incidental" and transmitted light, respectively. For a discussion of the derivation of this equation see p. 512 *et seq.*

To be valid as a basis for estimating vitamin A content, the absorption curve from which the extinction coefficient at the peak (325 mμ) is taken should be characteristic of the pure vitamin A absorption curve. Irrelevant absorption due to oxidation products of the vitamin or to other inactive substances may be evidenced by distortion of the vitamin A curve. This is more readily recognized when the curve is plotted without regard to concentration of the vitamin, i.e., on the assumption that the extinction coefficient at the maximum is equal to unity. The value at any other wavelength thus becomes an extinction ratio, i.e., the ratio of the extinction coefficient at that wavelength to that at the maximum (Fig. 275).

The conversion of the extinction coefficient (or "*E* value," as it has been abbreviated) to vitamin A units is effected by multiplying by a conversion factor derived from observed ratios of biological potency to *E* value in fish liver oils. In practice such conversion factors have been found to vary over a wide range in different laboratories, owing to differences in the oils themselves, as well as to the errors inherent in both the biological and instrumental methods employed. On the basis of improved analytical techniques as well as theoretical considerations, it is now commercial practice to regard the factor 1900 multiplied by the extinction coefficient at the maximum for the typical vitamin A curve as giving the best estimate of vitamin A unitage.

Determination of Preformed Vitamin A (Spectrophotometric Method): ***Principle.*** Vitamin A may be determined by measurement of the extinction coefficient of the characteristic absorption band in the ultraviolet region of the spectrum, whose maximum is in the region 325–328 mμ.

Procedure: Preparation of Unsaponifiable Extract. **Throughout the entire determination avoid exposure to actinic light preferably by using amber or red glassware. Into a saponification flask introduce a weighed sample containing 250–400 expected units of vitamin A (but not more than 1 g. fat or oil). Add 30 ml. ethanol and 3 ml. 50 per cent (w/w) potassium hydroxide and reflux in all-glass apparatus for 30 minutes. Cool, add 30 ml. water, transfer to separatory funnel and extract 4 times with 30-ml. portions of ethyl ether.[33] Collect the ether extracts in another separatory funnel and wash by gently swirling with 50 ml. water. Remove the aqueous layer and repeat washings with gradually more vigorous shaking until the aqueous layer is no longer alkaline to phenolphthalein. Evaporate the ether extract to about 50 ml. on a water bath, add 5–10 g. anhydrous sodium sulfate, stir and allow to settle. Decant into a 100-ml. volumetric flask and make up to 100 ml. with several ether rinsings of the sodium sulfate. (The final rinsing should give a negative antimony trichloride reaction.) Evaporate 10–25 ml. of the ether extract to about 2 ml. Remove from water bath and blow off remaining ether with a stream of nitrogen or *in vacuo*. Take up the residue in sufficient isopropanol (99 per cent purity) to give an expected concentration of 8–15 units of vitamin A per ml. (i.e., to show an absorbancy of 0.4–0.8 at 325 mμ).**

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[33] Peroxide-free and redistilled; discard first and last 10 per cent.

SPECTROPHOTOMETRIC DETERMINATION AND CALCULATION. Using quartz cuvettes, read the absorbancy of the isopropanol solution at 310, 325, and 334 mμ. The median wavelength is the absorption maximum of pure vitamin A alcohol in isopropanol, whereas the higher and lower are the wavelengths at which the absorbancy is $6/7$ (i.e., 0.857) of the maximum. The absorbancy ratios $A_{310/325}$ and $A_{334/325}$ are frequently found to deviate by more than 1 per cent from 0.857 due to the presence of non-vitamin-A-absorbing materials. Assuming that such irrelevant absorption is linear within narrow wavelength limits (e.g., $6/7$ above and below the peak) the correction procedure devised by Morton and Stubbs may be applied.[34] In simplified form[35] this has been adopted into official USP and AOAC vitamin A assays.

$$A \text{ (corrected)} = 7A_{325} - 2.625A_{310} - 4.375A_{334}$$

in which A is the absorbancy at the indicated wavelength.

$\dfrac{A\text{(corrected)}}{LC} \times 5.7 = \mu$g. vitamin A (per g. or other sample unit) in which L is the length of the absorption cell in cm., and C the fraction of the sample unit per ml. of solution read.

$$\mu\text{g. Vitamin A} \times 3.33 = \text{International (USP) units}$$

Comment. This method is applicable chiefly to fish liver oils where the vitamin is present in comparatively high concentrations. Though a more accurate value is obtained by conducting the determination on the unsaponifiable fraction, oils of high potency may often be assayed with little error by dissolving them directly in isopropanol. For the analysis of foods that have been enriched with vitamin A, a blank test of an unfortified sample or of a sample in which the vitamin A has been destroyed by selective ultraviolet irradiation[36] is advised.

Agreement between the basically unrelated spectrophotometric and colorimetric procedures may be regarded as highly presumptive of the accuracy of the nonbiological determination. In fact the official assays for vitamin-A-bearing materials in the U.S. Pharmacopeia and the Association of Official Agricultural Chemists specify that for the spectrophotometric assay to be valid the colorimetric test must agree within a ratio of 1.00 to 1.30.

Determination of Vitamin A and Carotene in Blood Serum** (**Colorimetric Method of Dann and Evelyn**):*[37] ***Principle. Vitamin A and carotene are extracted from serum with ethyl ether after saponification with alcoholic potassium hydroxide. Carotene is determined colorimetrically in the extract by photometric measurement of the light absorption of a choloroform solution at 440 mμ, and preformed vitamin A by measurement of the light absorption at 620 mμ of the blue color produced by reaction of the vitamin with antimony trichloride.

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[34] Morton and Stubbs: *Biochem. J.*, **41**, 525 (1947); **42**, 195 (1948).

[35] Oser: *Anal. Chem.*, **21**, 529 (1949).

[36] Little: *Ind. Eng. Chem., Anal. Ed.*, **16**, 288 (1944).

[37] Dann and Evelyn: *Biochem J.*, **32**, 1008 (1938). Vitamin A may also be determined in plasma or serum by the following procedure which avoids saponification: Add to the plasma slowly, while shaking, an equal volume of 95 per cent ethanol followed by 2 volumes of petroleum ether (b. pt. 40–60° C.). Stopper tightly and shake for 10 minutes. Centrifuge one minute at low speed. Evaporate an aliquot of the petroleum ether layer on a water bath (40° C.) in a stream of N_2 heating finally at 70° C. for a few seconds. The residue is taken up in ethanol-free chloroform for colorimetric determination. (Kimble: *J. Lab. Clin. Med.*, **24**, 1055 (1939); Yudkin: *Biochem. J.*, **35**, 551 (1941).)

Procedure. To 10 ml. of serum in a small flask add an equal volume of 95 per cent ethyl alcohol and 2 ml. of a 60 per cent aqueous solution of potassium hydroxide. Boil for 3 minutes. Pour the mixture into 10 ml. of water in a separatory funnel, and wash the flask with two 15-ml. portions of water, followed by two 25-ml. portions of ether, adding the washings to the separatory funnel. Shake the funnel vigorously for 1 minute, then allow the phases to separate. Discard the aqueous (lower) layer. Wash the ether phase by shaking vigorously with 10 ml. of water, then twice gently with 25 ml. of water. Filter the ether solution through a layer of anhydrous sodium sulfate on a sintered-glass filter. Wash the residue with 20 ml. of ether, combining the wash with the filtrate. With the aid of a stream of nitrogen, evaporate the ether solution to dryness on a hot water bath. Take up the residue in 10 ml. of chloroform in the absorption cell of a photoelectric colorimeter.

Measure the light absorption of the solution at 440 mμ, setting the instrument at 100 per cent transmittance with pure chloroform. Calibrate the instrument by measuring the absorption of standard solutions of C.P. β-carotene in chloroform containing 0.2, 0.4, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.5, and 3.0 μg. per ml. Plot photometric densities against concentrations of carotene. From the graph, determine the concentration of carotenoids per ml. in the chloroform extract of the unknown, and multiply this value by 100 to obtain the concentration per 100 ml. of serum.

Place the cell in a hot water bath and evaporate off the chloroform with the aid of a stream of nitrogen. The latter provides an inert atmosphere and prevents oxidation of vitamin A. Take up the residue in 1.7 ml. of chloroform. Place the cell in the photoelectric colorimeter equipped with a 620 mμ filter, set at 100 per cent transmittance with 10 ml. of a solution containing 1.7 ml. of chloroform and 8.3 ml. of antimony trichloride reagent (see p. 1124). Rapidly add 8.3 ml. of antimony trichloride reagent to the unknown and determine the maximal extinction. Calibrate the instrument by conducting the test on pure solutions of vitamin A in chloroform[38] containing 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, and 10.0 μg. per 1.7 ml.

CALCULATION. Plot photometric density against concentration of vitamin A per 1.7 ml. Determine the concentration in the chloroform extract of the unknown, and multiply this value by 10 to obtain the concentration of apparent vitamin A per 100 ml. of serum. This value must be corrected for the interference of carotenoids which react with antimony trichloride to produce a blue pigment. The color produced by 1 μg of preformed vitamin A is approximately equal to that of 20 μg. of β-carotene. Determine the true concentration of vitamin A by means of the formula

$$A - \frac{C}{20} = \mu\text{g. vitamin A per 100 ml. of serum}$$

where A is the concentration of apparent vitamin A and C that of carotene, both expressed per 100 ml. of serum.

Interpretation. The normal vitamin A level for blood serum is 15 to 60 μg (approximately 50 to 200 USP units) per 100 ml. The principal carotenoids in blood serum are carotene and xanthophyll, which are present in a relatively fixed proportion.[39] Under these conditions, measurement of total carotenoids, which is necessary for the determination of total vitamin A activity, would be a good practical index of carotene content. Chromatographic analysis, however, has demonstrated that the true carotene content varies from 10 to 50 per cent of the total carotenoid.[40] The normal total carotenoid range in blood serum is 100 to 300 μg. expressed as β-carotene.

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[38] Crystalline vitamin A acetate as contained in the USP Reference Standard. This ester is more stable than the free alcohol. On a molar basis the acetate and the free alcohol have equal chromogenicity with antimony trichloride.

[39] Clausen and McCoord: *J. Pediatrics*, 13, 635 (1938).

BIOLOGICAL METHODS

Since no single chemical or physical method for estimating vitamin A activity is sufficiently specific for all compounds and mixtures possessing vitamin A activity, the biological assay remains fundamentally the most reliable means for determining vitamin A potency and is the basis against which the more precise nonbiological procedures must be evaluated. Many of the limitations of bioassays are removed when they are planned to measure not only the response of animals to dosage of the test material but to known dosage of some standard of reference. By balancing the distribution of test animals among assay and control groups with respect to strain, litter origin, sex, age, and body weight, and standardizing the environmental factors, diet, feeding, dosing conditions, etc., it is possible to reduce the error of biological assay to a minimum. The assay can be set up according to a statistical design to permit control of these variables, and also to provide a measure of the limits of precision of the estimate and the extent to which individual factors such as sex, litter, etc. contribute to the total variance. An essential feature of bioassays in which responses are quantitatively graded to dosage is that the slope of the dose:response curve (plotted logarithmically) must be reasonably parallel to that of the reference curve. Otherwise the standard and unknown preparations are likely to be qualitatively different; e.g., the unknown may be contaminated with a substance that is toxic or inhibits absorption. The effect of departure from parallelism may be measured in an assay designed to permit factorial analysis of variance.

The curative method for assaying vitamin A is based on depleting young rats of their reserves of this vitamin and determining the relative growth responses of groups of these animals receiving daily supplements of the test material or of the vitamin A standard. The basal diet fed throughout the depletion and assay periods should be complete with respect to all nutrients required by the rat except vitamin A. Serious error may be caused by failure to include in the basal vitamin-A-free diet a sufficient amount of vitamin E (tocopherol), since the responses of the animals may be influenced by differences in the antioxidant content of the assay and reference materials.

The U.S. Pharmacopeia[41] method for vitamin A is a "rat-curative" assay and is most generally employed for the evaluation of food and pharmaceutical products. As described, however, it is a minimal-potency assay (i.e., it provides for one assay and one reference group), and therefore permits only the statement that the assay material contains "less than" or "not less than" the unitage of vitamin corresponding to the administered dosage level. The method may be adapted to quantitative estimation of actual potency by feeding two or more dosage levels of the assay material and a similar number of dosage levels of the standard of reference (USP Reference Vitamin A Standard). The dosage interval —i.e., the ratio of each dose to the next lower dose—is constant in both assay and reference groups. The resulting data may be computed according to the analysis of variance, as illustrated by the example in Appendix VI,[42] to give an estimate of potency, the standard error of this estimate, and, most important of all, an objective indication of whether the assay is a valid basis for such estimation.

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[40] Wohl: *Dietotherapy, Clinical Application of Modern Nutrition*, Philadelphia, W. B. Saunders Co., 1945, p. 225.

[41] XIVth Revision.

Biological methods have been described for vitamin A based on prevention rather than cure of the deficiency in rats, on keratinization of vaginal epithelium (which occurs at about the twenty-third day of depletion in young rats), and on the colorimetric determination of the vitamin A stored in the livers after controlled feeding periods.[43] The latter procedure is less adaptable than the curative rat-growth method to quantitative study since it requires the administration of massive doses of the vitamin.

The U.S. Pharmacopeia biological assays for vitamins A and D are given in full on pp. 1261 and 1264, respectively.

THE VITAMIN B COMPLEX

Since the vitamin concept was first postulated, the initial group of three vitamins has expanded to more than 20 by virtue of the discovery of the multiple nature of previously known vitamins or the recognition, from biological experiments, that as yet unknown nutrients must exist. Most outstanding in this regard has been the branching out of the vitamin B complex, a group of water-soluble factors usually associated in nature with thiamine (the original vitamin B), rich sources being liver, yeasts, and brans. The possibility that the "antiberiberi vitamine" of Funk (also known as the antineuritic factor or water-soluble B) might be a complex was suggested by the work of Emmett and Luros[44] on the basis of differences in susceptibility to heat destruction. Smith and Hendrick[45] showed that autoclaved yeast, in which the antineuritic vitamin was destroyed,

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[42] For comprehensive treatments and examples of the statistical design and interpretation of biological assays, reference may be made to the following works in this increasingly important field of biometrics: Fisher: *The Design of Experiments*, 2d ed. London, Oliver & Boyd, 1937; and *Statistical Methods for Research Workers*, 8th ed. London, Oliver & Boyd, 1941; Snedecor: *Statistical Methods Applied to Experiments in Agriculture and Biology*, 4th ed. Ames, Iowa State College Press, 1946; Emmens: *Principles of Biological Assay*, London, Chapman & Hall, 1948; Cochran and Cox: *Experimental Designs*, New York, John Wiley & Sons, Inc., 1950; Dixon and Massey: *Introduction to Statistical Analysis*, New York, McGraw-Hill Book Co., 1951; Moore, Cramer, and Knowles: *Statistics for Medical Students*, New York, The Blakiston Company, 1951; Youden: *Statistical Methods for Chemists*, New York, John Wiley & Sons, 1951; Bliss: *The Statistics of Bioassay with Special Reference to the Vitamins*, New York, Academic Press Inc., 1952; Finney: *Statistical Method in Biological Assay*, New York, Hafner Publishing Co., Inc., 1952.

[43] Guggenheim and Koch: *Biochem. J.*, **38**, 256 (1944); Foy and Morgareidge: *Anal. Chem.*, **20**, 304 (1948).

[44] Emmett and Luros: *J. Biol. Chem.*, **43**, 265 (1920).

[45] Smith and Hendrick: *U.S. Pub. Health Repts.*, **41**, 201 (1926).

TYPICAL MICROBIOLOGICAL DETERMINATIONS OF THE VITAMINS

Vitamin	*Extraction and Hydrolytic Procedure*	*Test Microorganisms*	*Vitamin Concentration*		*Response Measured*	*References for Preparation of Media and Details of Tests*
			at Half Maximum Growth	*at Maximum Growth*		
			µg. per 10 ml. tube			
Thiamine........	Heat 30 min. 100° C. 0.1 N H_2SO_4 and digest with pepsin and "Taka-Diastase"	*Lactobacillus fermentum* 36 (ATCC 9833)	0.02	0.04	Turbidity	*Analyst*, **74,** 340(1949)
Riboflavin.......	Autoclave 30 min., 15 lb., 0.1 N HCl	*Lactobacillus casei* (ATCC 7469)	0.06	0.20	Acidity	*U.S. Pharmacopeia* XIV
Niacin...........	Autoclave 30 min., 15 lb., 1.0 N H_2SO_4	*Lactobacillus arabinosus* 17-5 (ATCC 8014)	0.1	0.4	Acidity	*U.S. Pharmacopeia* XIV
Pantothenic acid.	Digest with intestinal phosphatase and liver enzyme	*Lactobacillus arabinosus* 17-5 (ATCC 8014)	0.03	0.20	Acidity	*J. Biol. Chem.*, **192,** 181 (1952)
Vitamin B_6......	Autoclave 60 min., 20 lb., 0.055 N or 2N H_2SO_4	*Saccharomyces carlsbergensis*	0.01	0.04	Turbidity	*Ind. Eng. Chem., Anal. Ed.*, **15,** 141 (1943); *J. Biol. Chem.*, **160,** 1 (1945)
Vitamin B_{12}......	(1) Aqueous (USP) (2) 15 min., 15 lb., pH 4.5 with fresh $NaHSO_3$	*Lactobacillus leichmanii* 313 (ATCC 7830)	0.00005	0.0002	Acidity	(1) *U.S. Pharmacopeia* XIV, 3rd Sup. (2) *J. Assoc. Off. Agr. Chem.*, **36,** 96 (1953)
Biotin...........	Autoclave 60 min., 15 lb., 6 N H_2SO_4	*Lactobacillus arabinosus*	0.0004	0.0015	Acidity	*Proc. Soc. Exp. Biol. Med.*, **56,** 95 (1944)

TYPICAL MICROBIOLOGICAL DETERMINATIONS OF THE VITAMINS—(Continued)

Vitamin	*Extraction and Hydrolytic Procedure*	*Test Microorganisms*	*Vitamin Concentration*		*Response Measured*	*References for Preparation of Media and Details of Tests*
			at Half Maximum Growth	*at Maximum Growth*		
Folic acids	Digest by specific enzymes from chicken pancreas	*Lactobacillus casei* (ATCC 7469)	0.0005	0.005	Acidity	*Arch. Biochem.*, **7**, 287 (1945)
p-Aminobenzoic acid	Autoclave 1 hr., 15 lb. 6 N H_2SO_4	*Neurospora crassa* 1633 (ATCC 9278)	0.015	0.04	Weight	*J. Biol. Chem.*, **148**, 281 (1943) modified
Choline	Autoclave 120 min., 15 lb., 1 N H_2SO_4	Cholineless mutant of *Neurospora crassa* No. 34486 (ATCC 9277)	6*	30*	Weight	*J. Biol. Chem.*, **150**, 325 (1943); *Science*, **101**, 674 (1945)
Inositol	Reflux 6 hrs., 120° C., 2.7 N HCl	*Saccharomyces carlsbergensis* (ATCC 9080)	3.0	8.0	Turbidity	*J. Bact.*, **47**, 434 (1944); *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943). Medium modified to include pyridoxine and exclude inositol

* Per 25 ml.

still possessed supplementary value for rats receiving adequate amounts of vitamin B from oats or in the form of a vitamin B picrate. Goldberger,[46] in 1926, recognized that rats required not only the antineuritic vitamin ("B *sensu stricto*") but also another factor which he called P-P (pellagra-preventive). In 1927 the Accessory Factors Committee of the British Medical Research Council recommended the symbols B_1 and B_2 for the heat-labile (antineuritic) and the heat-stable components, respectively. (For a time vitamin B_2 was called G in the United States.) The erroneous belief that the latter was identical with the pellagra-preventive factor was corrected when distinction was drawn between human pellagra and canine blacktongue, on the one hand, and so-called rat pellagra on the other. Between the time that Jansen and Donath,[47] in 1927, isolated the crystalline antiberiberi vitamin from rice bran and R. R. Williams[48] synthesized it and gave it the name *thiamine* in 1936, evidence accumulated for the increasing complexity of the vitamin B group.

Kuhn, György, and Wagner-Jauregg[49] isolated the fluorescent compound ovoflavin from egg white, designating it vitamin B_2. Their demonstration that this biologically active substance and similar flavins from milk and liver (lactoflavin and hepatoflavin) were identical, containing a ribose group attached to an isoalloxazine ring, caused them to adopt the common name *riboflavin* for vitamin B_2.

Evidence for the existence of additional growth and antidermatitis factors for rats, pigeons, and chicks prompted the continued search for new vitamins in the B group, which in the years since 1927 has culminated in the identification and synthesis of at least ten additional vitamins. From the standpoint of human nutrition, one of the most significant is *nicotinic acid* (now called *niacin*), which was identified by Elvehjem, Madden, Strong, and Woolley,[50] in 1937 as the canine blacktongue factor. This compound had been known for many years and was actually isolated from rice polishings by Funk[51] in his effort to isolate "vitamine B." The therapeutic trial of nicotinic acid in human pellagra was rewarded with prompt and dramatic success.

Clinical experience with beriberi or pellagra has emphasized that these diseases are often associated with deficiencies of other nutrients than thiamine or niacin alone. Therapeutic administration of individual crystalline vitamins often reveals the presence of underlying deficiencies which respond to supplementary dosage with other vitamins of the B group or with rich sources of the vitamin B complex such as yeast or liver. In this connection it is significant that the relative proportions of the various members of the vitamin B group as found in natural sources, both plant and animal, are quite variable; for example, seeds and legumes are relatively higher in thiamine than in riboflavin, whereas in milk and leafy vegetables the reverse is true. Processing by heat, exposure to light, or other destructive influences effect still further variations in the quantitative relationship of the B vitamins.

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[46] Goldberger, Wheeler, Lillie, and Rogers: *U.S. Pub. Health Repts.*, **41**, 297 (1926). Goldberger and Lillie: *Ibid.*, **41**, 1025 (1926).

[47] Jansen and Donath: *Mededeel. Dienst. Volksgezondheid in Nederland.-Indië*, **16**, 186 (1927).

[48] Williams: *J. Am. Chem. Soc.*, **58**, 1063 (1936).

[49] Kuhn, György, and Wagner-Jauregg: *Ber.*, **66**, 317, 576 (1933).

[50] Elvehjem, Madden, Strong, and Woolley: *J. Am. Chem. Soc.*, **59**, 1767 (1937).

[51] Funk: *J. Physiol.*, **43**, 395 (1911); **46**, 173 (1913).

Bacteria present in the mammalian intestinal tract are capable of synthesizing various vitamins of the B group, noteworthy among them being biotin, folic acid, and pantothenic acid. Factors which influence such bacterial growth—e.g., nutrients in the diet of the host or bacteriostatic medication (like the sulfonamides)—may depress intestinal synthesis sufficiently to induce secondary deficiencies.

The use of microbiological assay procedures has aided materially in the discovery of additional factors in the vitamin B group which are essential for the nutrition of certain microorganisms or laboratory animals but whose role as vitamins for man is not yet established. Among these factors are *p*-aminobenzoic acid, thioctic (lipoic) acid, *L. citrovorum* factor, *L. bulgaricus* factor, and vitamin B_T (the latter required by the mealworm *Tenebrio molitor*). Examples of microbiological assays are given under Riboflavin, Pantothenic Acid, Niacin, Vitamin B_6, and Pteroylglutamic Acid. In principle, these methods are identical with the procedures for amino acid assay described in Chapter 33 and illustrated on p. 1064. A table outlining the conditions for extraction, hydrolysis, and optimal concentration for microbiological vitamin assays by selected methods is given on p. 1131.

THIAMINE

The term "deficiency disease" was first applied to a condition known as beriberi, which was common in southeastern Asia and the islands of the Pacific Ocean. The similarity in pathology between this disease and polyneuritis observed in fowls restricted to a diet of polished rice, both of which could be cured by feeding the "silverskin" (i.e., pericarp and germ) of the grain, prompted Eijkman, in the Dutch East Indies, to investigate the subject from the nutritional standpoint. Eijkman produced beriberi in fowls on a diet of polished rice and prevented the syndrome by dietary means. His classic studies, reported in 1896–1897, paved the way for a host of other investigations which culminated in the formulation of a vitamin hypothesis. Besides those Oriental countries where polished rice is the main article of diet, other places in which beriberi has been observed are prisons or asylums, localities where the diet is apt to be restricted or faulty, and war-stricken countries.

After numerous attempts to isolate the vitamin by methods involving silver or lead precipitation, crystallization of a picrate, adsorption, etc., the vitamin was finally crystallized from rice polishings by Jansen and Donath in 1926, in the very laboratories in which the original studies were conducted by Eijkman, who was able, after thirty years, to confirm the antineuritic activity of the crystalline vitamin hydrochloride. A decade later the structure and synthesis of the vitamin were announced by R. R. Williams and his co-workers.

Clinical cases of thiamine deficiency in the Western Hemisphere are rare today. Subclinical cases of deficiency are probably more prevalent, arising from a limited intake of the vitamin or from increased requirements such as are observed in pregnancy or lactation. The neuritis observed in cases of chronic alcoholism has been claimed to result from diminished intake of food as well as insufficient thiamine to metabolize the alcohol consumed.

Physiological and Clinical Aspects of Thiamine.[52] Thiamine is essential for the growth and metabolism of all animals as well as of many plants and microorganisms. Deficiencies are characterized by a variety of symptoms and clinically are often complicated by the effects of lack of other nutrients.

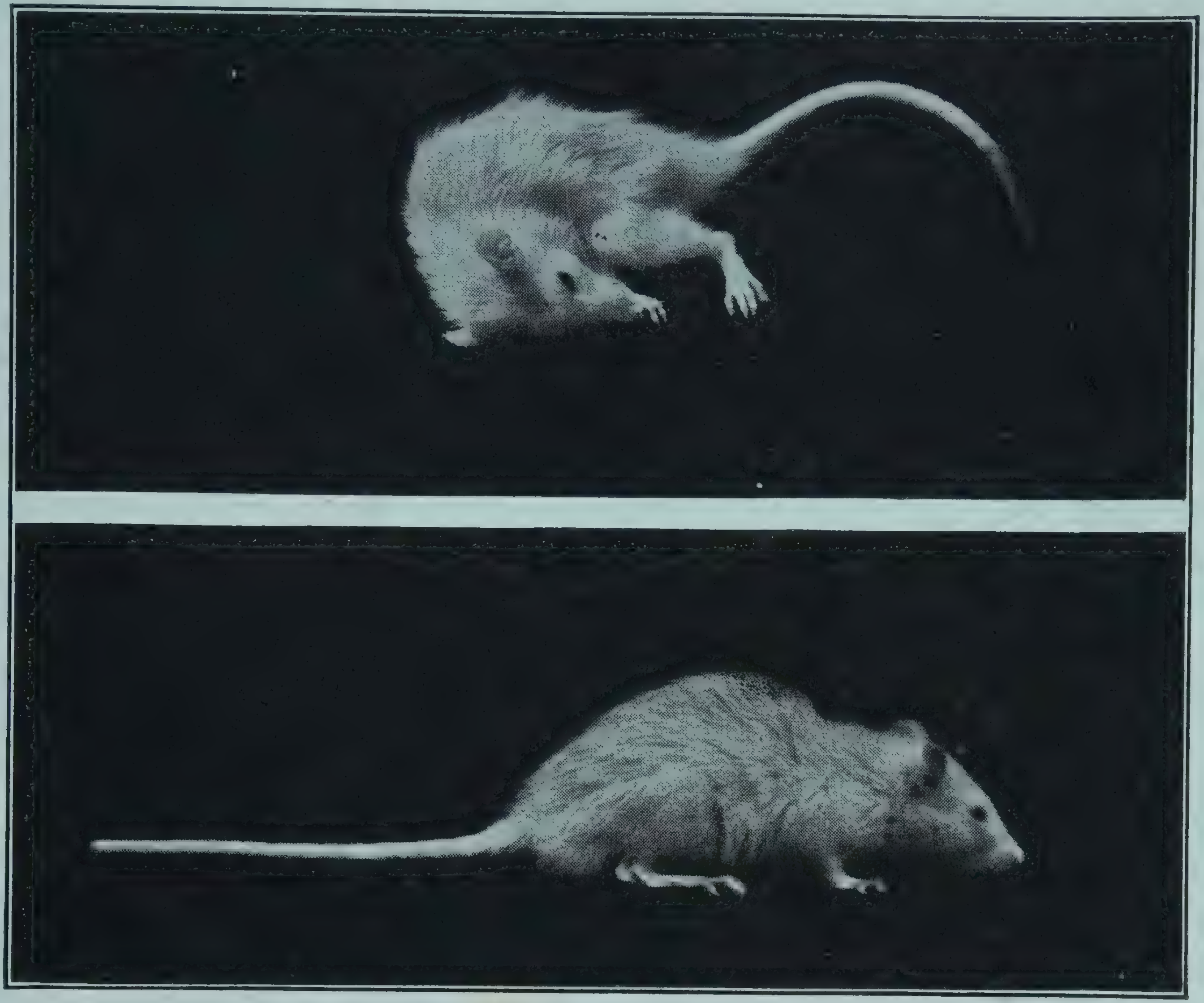

FIG. 276. EFFECT OF THIAMINE DEFICIENCY AND SUBSEQUENT CHANGE TO AN ADEQUATE DIET.

The spastic paralysis shown in the upper figure was cured (*below*) about 24 hours after addition of thiamine.

Smith and Munsell: *U.S. Dept. Agr. Circ. No.* 84, 1929. Courtesy, The Bureau of Home Economics, U.S. Department of Agriculture.

In thiamine deficiency (avitaminosis B_1) a form of peripheral neuritis is manifested affecting both the sensory and motor nerves. During the early stages neuralgia and cramps of the calf muscles are common; as the condition advances the thigh muscles become weak and toe- and foot-drop develop along with hypesthesia. The acute disease, beriberi, may be of the dry type, in which cachexia, numbness, and paralysis are the primary symptoms, or of the wet type, associated with marked ("pitting") edema and paresthesia of the extremities. In animals the symptoms are loss of muscular coordination, spastic movements, retraction of the head (opisthotonos), and paralysis (see Fig. 276). Recovery from the symptoms of polyneuritis is very rapid when thiamine is administered, especially by injection.

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[52] Cowgill: "The Physiology of Vitamin B_1," in *The Vitamins*, Chicago, American Medical Association, 1939; Vedder: "The Pathology of Beriberi," *ibid.;* Strauss: "The Therapeutic Use of Vitamin B_1 in Polyneuritis and Cardiovascular Conditions," *ibid.*

Debility and progressive decline in weight are observed early in vitamin B_1 deficiency as a result of anorexia (loss of appetite), one of the most striking symptoms of this deficiency disease. The loss of appetite, which is the main cause of growth failure, has been attributed to a general systemic disturbance rather than to diminished secretory functions of the digestive glands or to reduced gastric motility, although diminished motor function of the gastrointestinal tract is observed clinically in thiamine deficiency.

The rapid restoration of appetite following thiamine supplementation of avitaminotic subjects has been regarded as indicating a specific appetite-stimulating function of the vitamin. The anorexia of thiamine deficiency, however, is more probably explained by the impaired carbohydrate metabolism of cellular tissue in general. Attempts to explain the loss of appetite on the basis of lowered secretory or motor function or diminished basal metabolic rate have not succeeded.

In polyneuritis pathological changes in the tissues and organs of the body include cardiovascular and neural disturbances and atrophy of the endocrine glands and other vital organs, but hypertrophy of the adrenals. Prolonged deficiency results in cardiac failure and death. An outline of the clinical symptoms of thiamine deficiency is given in the American Medical Association syllabus on p. 1289.

Degeneration of the myelin sheaths of peripheral nerves and also of the ganglion cells of the brain and spinal cord is produced in experimental polyneuritis, but since similar findings are observed in starvation, even when the supply of thiamine is adequate, it is preferable to regard the primary neurological effect of the avitaminosis as a functional defect concerned with the physiology of the neurons.[53] The rapid recovery from neurological symptoms which follows thiamine therapy supports this view.

In 1937 Lohmann and Schuster[54] isolated from yeast a crystalline coenzyme, cocarboxylase, the pyrophosphate ester of thiamine. When this compound is bound to a specific protein (apoenzyme) from yeast, and a magnesium ion, an active enzyme (carboxylase) is formed which catalyzes the decomposition of pyruvic acid to acetaldehyde and carbon dioxide. Unphosphorylated thiamine has no cocarboxylase activity, although it can stimulate the action of cocarboxylase in alkali-washed yeast preparations. The pyrimidine portion of the vitamin with an intact amino group has similar properties. Substitution of the magnesium by other divalent ions or combination of the cocarboxylase with different specific apoenzymes may result in more than one form of the enzyme and in somewhat diminished biological activity.

Free thiamine is phosphorylated *in vivo* to cocarboxylase (or diphosphothiamine) by liver and kidney tissue and to some extent by muscle and brain. The phosphorylating agent appears to be adenosine-5′-triphosphate (ATP).

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[53] **Wolbach:** *J. Am. Med. Assoc.*, **108**, 7 (1937).

[54] **Lohmann and Schuster:** *Naturwissenschaften*, **25**, 26 (1937).

In animal tissues, cocarboxylase plays a role in various reactions involving principally the decarboxylation of pyruvic and other keto acids. In the brain, cocarboxylase participates in the anaerobic dismutation of pyruvate to lactate and acetate, and their subsequent oxidation to carbon dioxide and water. In liver and other tissue cells, cocarboxylase is involved in the conversion of pyruvate to oxalacetate which combines oxidatively and irreversibly with another molecule of pyruvate to enter the tricarboxylic acid cycle. The oxidative decarboxylation of ketoglutarate to succinate and of pyruvate to acetoacetate, and the conversion of pyruvate to the acetyl group of acetyl–coenzyme A (see p. 1188), are additional examples of reactions found to be mediated by cocarboxylase.

It is not an exaggeration therefore to say that thiamine occupies a key position in at least the terminal stages of carbohydrate metabolism. In advanced thiamine deficiency both pyruvate and lactate accumulate in the blood whereas glycogen increases in the liver and heart muscle. Rats subsisting on diets of glucose or casein alone survive twice as long when thiamine is added.

Diphosphothiamine may be involved in the synthesis of acetylcholine and in the control of its hydrolysis *in vivo*. The activity of choline esterase of serum is strongly inhibited by cocarboxylase.

The average American diet has been estimated to contain 1.3 mg. of thiamine per day. Prior to bread and flour enrichment, the value was 0.8 mg. Moderate thiamine deficiency may be the root of many ill-defined nutritional disturbances, especially when anorexia is a symptom. Such cases respond readily to the therapeutic test; i.e., supplementation of the diet. The thiamine requirement is a function of sex, body weight, muscular activity, and other conditions. Diet, especially the intake of nonfat calories, is an important factor. The recommended daily allowances of thiamine are shown in the table on p. 1108. The remarkable tolerance for thiamine is indicated by the fact that 500 mg. have been taken daily for a month by normal people, without any objective symptoms. It is of interest in this connection that peripheral neuritis in chronic alcoholism has been attacked therapeutically on the hypothesis that the condition is due partly to a disturbance of the vitamin B_1:nonfat calorie ratio.

It has been shown that lactating rats require an additional quota of antineuritic vitamin, in the absence of which the mother will neglect or destroy the litter. The vitamin may be supplied directly to the young, or indirectly through the mother's milk.[55]

Thiamine and its compounds are the only naturally occurring substances having vitamin B_1 activity for the higher species. Certain synthetic analogs have less biological potency; e.g., the compound having a methyl group in the 6-, rather than in the 2- position in the pyrimidine nucleus; the 2-ethyl, instead of the 2-methyl, compound is active for

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[55] Sure: *J. Biol. Chem.*, **76**, 685 (1928); Sure and Walker: *J. Biol. Chem.*, **91**, 69 (1931); Macy, Outhouse, Graham, and Long: *J. Biol. Chem.*, **73**, 189 (1927).

Phycomyces; thiamine disulfide, a possible intermediate in the metabolism of thiamine, obtainable by mild oxidation of the vitamin, has full activity.

Thiamine may be inactivated both *in vitro* and *in vivo* by an enzyme, thiaminase, present in certain fish and shellfish (see p. 1284). The vitamin is hydrolyzed to 2-methyl-6-amino-5-hydroxymethyl pyrimidine and 4-methyl-5-hydroxyethylthiazole.

Pyrithiamine, a mixture of derivatives which include the true pyridine analog of thiamine (i.e., 2-methyl-3-β-hydroxyethylpyridine in place of the thiazole moiety), and oxythiamine, in which oxygen replaces the sulfur atom, are powerful thiamine antagonists (see Chapter 36).

Storage and Synthesis of Thiamine. The available evidence seems to indicate little capacity on the part of the animal body to store thiamine. It occurs both free and phosphorylated, somewhat higher concentrations being present in the heart, liver, and kidneys than in muscles and brain. It is possible to increase the thiamine content of the tissues by dietary means; however, their storage capacity is so limited that even under these circumstances only a few weeks' reserve can be maintained. It is thus important that the daily diet include an adequate supply of thiamine.

Whereas certain microorganisms can synthesize the vitamin, others require an external source; some utilize the thiazole and pyrimidine portions—e.g., *Phycomyces blakesleanus*—whereas others can synthesize one moiety and combine it with the other obtained from an external source. Thiamine is synthesized by all higher plants, though to only a limited extent in the dark.

Though all animals require thiamine, sheep and cattle do not need an external supply since the vitamin is synthesized in sufficient amount by bacteria in the rumen. Though thiamine is known to be synthesized by intestinal flora in rats and humans, the nutritional importance of this contribution is not known. Difficulties encountered in the production of experimental thiamine deficiency in humans subsisting on a diet low in thiamine have been attributed to the intestinal synthesis.

Thiamine and cocarboxylase have the same biological activity for higher animals. Thiamine can be phosphorylated by live yeast, by dried yeast, or by a phosphatase obtainable in the presence of hexosediphosphate, adenosinetriphosphate, and a specific protein. The vitamin may also be phosphorylated by certain bacteria. Liver and kidney, and to some extent muscle and brain, can perform the same function.

Distribution of Thiamine.[56] This vitamin is essentially of plant origin. There is no proof of its synthesis by the animal body proper although it is synthesized to some extent by bacteria in the intestinal tract. Its presence in such products as milk, eggs, and liver depends on the dietary supply of the animal. Of the various meats used for human consumption, pork muscle is the richest in thiamine. The ability to synthesize thiamine is possessed by plants and by lower forms of life; e.g., bacteria, molds, and yeasts. The latter are a potent natural source of this factor, as well as of other components of the vitamin B complex.

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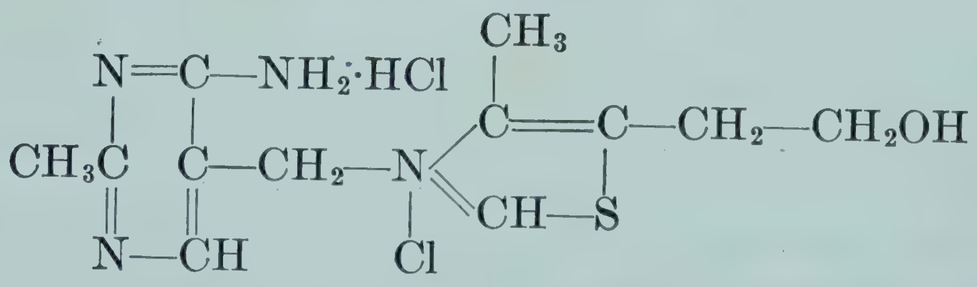
[56] For thiamine values in foods, see Appendix III.

Thiamine is widely distributed in the plant world, seeds, leaves, roots, stems, and fruits being recognized sources. In cereal grains as a class, thiamine is found in highest *concentration* in the germ or embryo, less in the bran, and least in the endosperm. The amount of thiamine in a milled grain such as flour depends on the proportion of each part of the grain present. Leaves and grasses (e.g., spinach, alfalfa, timothy hay, etc.) are rich sources of thiamine as are also beans, nuts, fruits, milk and egg yolk.

Vitamin B_1 may occur in natural materials as free thiamine, as a protein complex, as pyrophosphoric acid complex (cocarboxylase), and as a phosphorus-protein complex. In milk, the vitamin is present both as free thiamine and as the vitamin-protein complex. In brain and liver, it is found as cocarboxylase and as its protein complex. In wheat, thiamine occurs in the free form. In blood, it is present as the pyrophosphate in the cells and as free vitamin in the serum. Thiamine is excreted in the free form in the urine.

Chemistry of Thiamine. The efforts of many investigators to isolate pure crystalline thiamine from natural sources culminated in the work of Jansen and Donath (*loc. cit.*). They adsorbed an acid extract of rice polishings on clay, eluted with $Ba(OH)_2$, and alternately precipitated (first with silver nitrate, then with phosphotungstic or silicotungstic acid) and decomposed with acid, finally obtaining a platinic chloride precipitate from which a crystalline thiamine hydrochloride was prepared. The procedure yielded about 1 part of the vitamin from 3,000,000 parts of rice polishings.

Analysis of the crystalline vitamin led to its synthesis by Williams and his co-workers in 1936,[57] which was soon followed by large-scale commercial production. Thiamine chloride is 3-(4′-amino-2′-methylpyrimidyl-5′-methyl)-5-β-hydroxyethyl-4-methylthiazolium chloride. Its hydrochloride, commonly called thiamine chloride, is



Thiamine chloride hydrochloride
($C_{12}H_{17}N_4SOCl{\cdot}HCl$)

Thiamine hydrochloride is a white crystalline solid. The crystals are stable at 100° C. for 24 hours but decompose when heated to the melting point of 249° C. The compound is hygroscopic and dissolves in water to form an acid solution which is optically inactive. One gram dissolves in 1 ml. of water, in 18 ml. of glycerol, in 100 ml. of 95 per cent alcohol, or in 315 ml. of absolute alcohol. It is insoluble in ether, acetone, chloroform, and benzene. The vitamin has a characteristic yeasty odor and taste.

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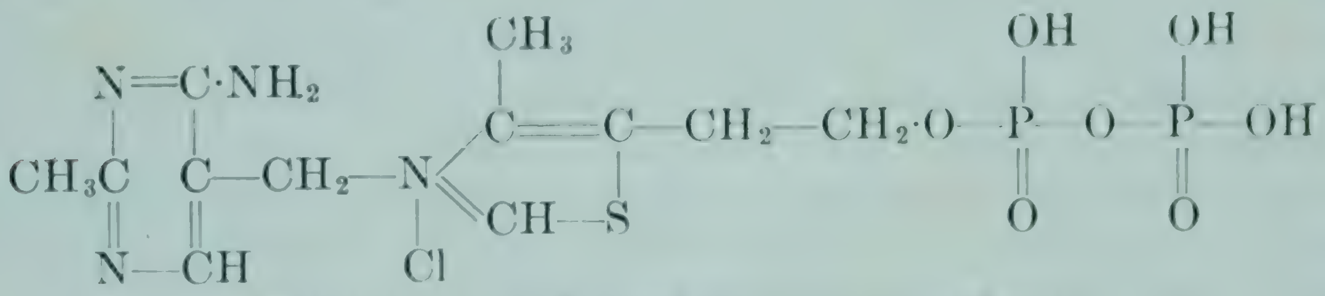
[57] Williams: *J. Am. Chem. Soc.*, **58**, 1063 (1936); Cline, Williams, and Finkelstein: *J. Am. Chem. Soc.*, **59**, 1052 (1937); Williams: *Ind. Eng. Chem.*, **29**, 980 (1937). The synthesis of thiamine was also accomplished, at about the same time, by Todd and Bergel (*J. Chem. Soc.*, p. 364 (1937)) and by Andersag and Westphal (*Ber.*, **70**, 2035 (1937)).

Thiamine is a basic substance forming insoluble compounds with picric, phosphotungstic, and tannic acids and with salts of the noble metals. It is adsorbed from acid or neutral solution by fuller's earth, silica gel, or "Norit," and may be eluted by alkalinization or treatment with quinine.

As the pH increases beyond neutrality, thiamine becomes unstable, especially at elevated temperatures. Heating at 100° C. in acid or neutral solution for one hour has little destructive effect but at higher temperatures gradual destruction takes place. However, at pH 3.5 or less the vitamin may be autoclaved at 120° C. with little loss. Oxidation is not the primary factor in this decomposition. Dried yeast when heated in an autoclave for several hours at 120° loses its thiamine activity but retains practically all of its riboflavin, this difference in thermolability being one of the earliest methods applied to the differentiation of the vitamin B complex.

The thiamine in foods is stable at cooking temperatures, though a considerable fraction is extracted and often discarded in the cooking water. In neutral or alkaline solution, thiamine exhibits spectrophotometric absorption maxima at 235 and at 267 mμ. Below pH 5.5 there is a single band at 246 mμ.

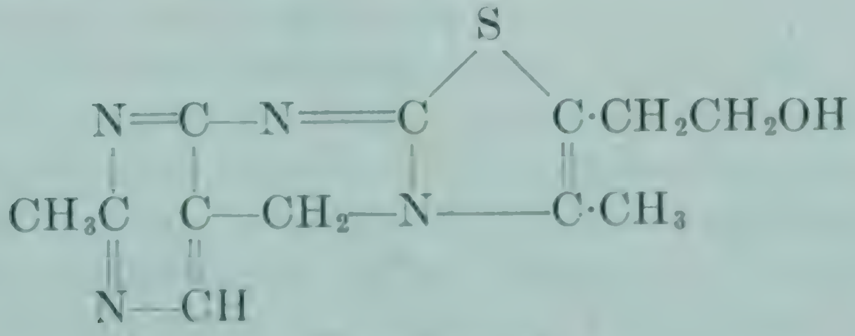
The principal ester of thiamine is the pyrophosphate, known as cocarboxylase or diphosphothiamine.



Thiamine pyrophosphate (cocarboxylase)

This compound differs from the free base in that it functions as a coenzyme in the decarboxylation of pyruvic acid. It is a white, crystalline compound melting at 242–244° C., and resembles thiamine itself in its susceptibility to mild oxidation and sulfite-cleavage.

Thiamine and its pyrophosphate are destroyed when treated with sulfite or nitrite owing to cleavage of the two-ring structure of the molecule. Both thiamine and cocarboxylase, when treated with mild oxidizing agents like potassium ferricyanide in alkaline medium, are converted to thiochrome, a blue fluorescent compound.



Thiochrome

Likewise both react with diazotized *p*-aminoacetophenone to produce red dyes. The reaction products obtainable from the free vitamin may be distinguished from those derived from the pyrophosphate by extraction of the former with organic solvents in which the latter are insoluble. The thiochrome or diazotization reactions serve as the basis for analytical determination of the vitamin.

DETERMINATION OF THIAMINE

Reliable chemical methods for the determination of thiamine are based on (*a*) measurement of the fluorescent compound, thiochrome, obtained by mild oxidation, and (*b*) the more specific but less sensitive diazotization reaction of Prebluda and McCollum. Colorimetric assays of various extracts and concentrates have been found to correlate well with bioassay results, whereas thiochrome values are somewhat low.[58] Thiochrome values have also been found to be low when compared with the results obtained in yeast fermentation studies and in rat-curative tests.[59]

For the chemical tests, the thiamine must first be extracted quantitatively from foods and biological materials by boiling with dilute acids. The vitamin is then freed from its natural complexes by the action of enzyme preparations containing phosphatases. This step, by hydrolyzing carbohydrates, also facilitates extraction. In order to accomplish complete extraction from materials rich in protein, digestion with proteolytic enzymes like pepsin is also employed. The extract of the free vitamin is then purified by passage of the solution through a column of zeolite, a synthetic adsorbent which removes thiamine and allows the passage of many undesirable materials. Reducing agents, heavy metals, and other compounds which interfere with the thiochrome and colorimetric reactions are discarded in the eluate. The vitamin is then eluted from the zeolite column in a relatively pure form with an acidified solution of potassium chloride.

In the thiochrome procedure the vitamin is oxidized by an alkaline solution of potassium ferricyanide to thiochrome. This fluorescent compound is extracted with isobutanol and determined photometrically. In order to correct for the presence of other fluorescent compounds which may be present in the final extract, a blank test is conducted in which the ferricyanide is omitted. Since the conversion to thiochrome is not quantitative and other oxidation products which do not fluoresce may be formed, care must be exercised to standardize the oxidation procedure and to employ a fixed amount of ferricyanide. Though thiamine pyrophosphate (cocarboxylase) also yields a blue fluorescent compound on oxidation with ferricyanide, the product is not extractable by butanol.

The assumption that the background fluorescence is independent of the treatment with ferricyanide may account for low values by the thiochrome procedure. This assumption is invalid when N^1-methylnicotinamide is present,[60,61] since this compound fluoresces in the thiamine blank, but not when treated with ferricyanide. N^1-Methylnicotinamide is a metabolite of niacin which, like thiamine, is adsorbed by and eluted from zeolite. It is partly responsible for erroneous results obtained in the assay of urine by the thiochrome method. In the analysis of materials that yield clear solutions and low blanks, preliminary tests may justify omission of the adsorption-elution step (in both the standard and unknown). Examples of such materials are certain pharmaceutical preparations, enriched flour, meat extracts, and milk.

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[58] Brown, Hartzler, Peacock, and Emmett: *Ind. Eng. Chem., Anal. Ed.*, **15**, 494 (1943).

[59] Hennessy, Wapner, and Truhlar: *Ind. Eng. Chem., Anal. Ed.*, **16**, 476 (1944).

[60] Najjar and Ketron: *J. Biol. Chem.*, **152**, 579 (1944).

[61] Mason and Williams: *J. Biol. Chem.*, **146**, 589 (1942); **140**, 417 (1941).

In the colorimetric procedure, the eluate from the zeolite is first added to a solution of phenol in alcohol to increase the sensitivity of the test. The solution is then made alkaline and diazotized *p*-aminoacetophenone is added. The mixture is allowed to stand while the red dye develops. The latter is then extracted selectively by xylene which leaves behind in the aqueous phase dyes produced by the coupling of the reagent with phenol, phosphorylated thiamine (if present), acid-base indicators, and other interfering compounds. Results obtained by the colorimetric procedure agree well with those obtained by biological assay.

Since many microorganisms require an external supply of thiamine for growth, microbiological methods may be employed for the determination of this vitamin. Caution should be exercised in the choice of microorganisms, however, since some can utilize portions of the thiamine molecule, or degradation products of the vitamin which are not biologically active for higher organisms. Successful microbiological assays have been conducted employing *Lactobacillus fermentum* 36, the growth of which, under the conditions of the test, is not stimulated by the pyrimidine or thiazole portions of the thiamine molecule, either alone or together. Results obtained by yeast fermentation methods are erroneous if the pyrimidine portion is present, since it stimulates yeast fermentation. Correction for this interference may be made by conducting a test on a sulfite-treated sample in which the thiamine is inactivated.[62,63]

Cocarboxylase may be determined specifically and directly by measurement of the carbon dioxide produced by decarboxylation of pyruvic acid by yeast cells previously washed free of cocarboxylase.[64]

Human thiamine deficiency, both clinical and subclinical, may be diagnosed by several biochemical procedures. These involve measurement of the concentration of thiamine, cocarboxylase or pyruvic acid in the blood or urine. Since the levels of these compounds may fluctuate widely depending on the dietary intake for several days immediately preceding the test, a more reliable diagnosis is made when the response to a test dose of the vitamin is measured. A simple and reliable diagnostic test[65,66] involves colorimetric measurement (see p. 1144) of the urinary excretion of thiamine in the 4-hour period following intramuscular injection of 0.35 mg. of the vitamin per square meter of body surface, 12 hours after the last meal. Normal individuals excrete more and deficient subjects less than 50μg. of thiamine during the 4-hour period.

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[62] Schultz, Atkin, and Frey: *J. Am. Chem. Soc.*, **59**, 948, 2457 (1937); **60**, 1514 (1938); *J. Biol. Chem.*, **136**, 713 (1940); *Ind. Eng. Chem., Anal. Ed.*, **14**, 35 (1942).

[63] Deutsch: *J. Biol. Chem.*, **152**, 431 (1944).

[64] Lohmann and Schuster: *Biochem. Z.*, **294**, 188 (1937).

[65] Hochberg and Melnick: *J. Biol. Chem.*, **156**, 53 (1944).

[66] Melnick and Field: *J. Nutrition*, **24**, 131 (1942).

CHEMICAL METHODS

1. Modification of the Fluorometric Method of Hennessy and Cerecedo:[67] ***Principle.*** The thiochrome method for the determination of thiamine involves extraction of the vitamin, dephosphorylation, purification by adsorption-elution, and oxidation. The fluorescent thiochrome is extracted with isobutanol and determined fluorometrically. For a general discussion of fluorometry see Chapter 23.

Procedure: Preparation of Sample. **Extract a sample containing not more than 2 g. of solids by refluxing for 0.5 hour with 75 ml. of 0.1 N sulfuric acid. Cool to 40° C. If the material is rich in protein, add 0.5 g. of pepsin and incubate over night at 37° C. Bring to 100° C. and then cool. Add 10 ml. of 1.8 M sodium acetate and adjust the pH to 4.5 if necessary. Add 0.5 g. of "Taka-Diastase" and incubate overnight at 37° C., or 3 hours at 45–50° C. Cool. Dilute to 100 ml. with distilled water, and filter.**

Pass an aliquot of the filtrate containing approximately 3–5 μg. of thiamine through a base-exchange tube[68] **containing a column of activated zeolite.**[69] **Wash the column twice with 20-ml. portions of distilled water. Elute the vitamin with 25 ml. of 25 per cent potassium chloride in 0.1 N hydrochloric acid. Mix the eluate.**

Pass a standard solution containing 5 μg. of thiamine in acidulated water (pH 4.5) through a separate column, and elute as above.

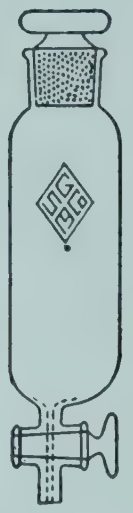

FIG. 277. SEPARATORY-CENTRIFUGE TUBE.

Courtesy, Scientific Glass Apparatus Co., Bloomfield, N. J.

Fluorometric Procedure. **Pipet a 5-ml. aliquot of the eluate into each of two 30-ml. separatory funnels. Specially prepared separatory centrifuge tubes, (see Fig. 277) are particularly valuable for this purpose. To one, the test (*T*), add 3 ml. of potassium ferricyanide solution.**[70] **Add 16.5 ml. of isobutanol to both, then 3 ml. of 15 per cent sodium hydroxide to the other, the blank (*B*). Shake the funnels for 1.5 minutes, centrifuge, and discard the aqueous phases. Clarify the isobutanol layers by shaking with approximately 1 g. of anhydrous sodium sulfate, transfer to cuvettes, and read in a fluorometer equipped with appropriate filters.**[71] **Use an aqueous solution of quinine sulfate containing 0.2 μg. per ml. in 0.1 N sulfuric acid to check the setting of the instrument.**

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[67] Hennessy and Cerecedo: *J. Am. Chem. Soc.*, **61**, 179 (1939); Hennessy: *Ind. Eng. Chem., Anal. Ed.*, **13**, 216 (1941).

[68] The base-exchange tube contains a reservoir at the upper end having a capacity of approximately 30 ml., followed by a tube of 5 to 6 mm. internal diameter approximately 14 cm. long. At the lower end is a capillary of such diameter that when the tube is charged, the rate of flow will be approximately 1 ml. per minute.

[69] Approximately 50 mesh "Decalso." Activate the zeolite by stirring with four 10-volume portions of 3 per cent acetic acid for 10 minutes each. Between the second and third acid wash, treat for 15 minutes with 5 volumes of 25 per cent potassium chloride solution. Wash the zeolite with water till free of chloride, then with alcohol, and ether. Dry in air and store in a sealed bottle. Place a pledget of glass wool over the upper end of the capillary, and pour a water suspension of 2 g. of the activated zeolite into the tube.

[70] Dissolve 30 mg. of potassium ferricyanide in 100 ml. of 15 per cent sodium hydroxide.

[71] The fluorometer should be equipped with an ultraviolet light source and a filter with a transmission peak at 3700 Å. Between the glass cuvette and the photocell, insert a secondary filter with a transmission peak at 4600 Å. Since thiochrome is unstable to light, make the measurements rapidly and in a semidark room.

CALCULATION. Calculate the thiamine content of the sample employing the formula:

$$\frac{T_u - B_u}{T_s - B_s} \times \frac{1}{5} \times \frac{25}{A} \times \frac{100}{G} = \mu\text{g. thiamine per g. of sample}$$

T_u equals the galvanometer reading obtained with the isobutanol extract of the unknown treated with ferricyanide; T_s is the corresponding value obtained with the standard; B_u and B_s are the galvanometer readings obtained in the blank tests conducted on the unknown and standard respectively; A is the volume of the unknown solution passed through the column; and G is the weight of sample taken for analysis.

2. *Colorimetric Method of Hochberg, Melnick, and Oser:*[72] *Principle.* The red pigment formed in the reaction between thiamine and diazotized *p*-aminoacetophenone is measured in a photoelectric colorimeter. The method involves the preparation of a clear extract of the free vitamin, adsorption on and elution from a zeolite column, reaction with diazotized *p*-aminoacetophenone, extraction of the red pigment with xylene, and comparison with a standard similarly treated. The method is applicable to the determination of thiamine in urine. Here the extraction and enzymic hydrolysis are omitted, and the urine samples, after adjustment of the pH to 4.5, are passed directly through the zeolite column.

The use of large samples for analysis makes the colorimetric method sufficiently sensitive for the determination of thiamine in most products. However, it is not recommended for the assay of materials rich in protein but low in thiamine. Because large samples are taken for analysis, high concentrations of adsorbable amino acids are released which interfere with the retention of thiamine on the zeolite column.

Procedure: Preparation of the Extract. Weigh out a sample containing at least 20 μg. of thiamine.[73] Reflux for 30 minutes with 150 ml. of 0.05 N sulfuric acid, cool to 50° C., and add 10 ml. of 1.8 M sodium acetate solution. Adjust the pH to 4.5 if necessary. Add 1 g. of "Taka-Diastase," and incubate the suspension 3 hours at 45–50° C. or preferably overnight at 38° C. Cool and dilute to 200 ml. Filter.

The apparatus used for the purification and concentration of the extract is shown in Fig. 278 (p. 1175). A glass funnel is sealed to the top of a condenser. This is connected by means of a ground glass joint to a zeolite filter. The latter is a tube having an internal diameter of 8 mm. for a length of 12 cm. beyond the joint, followed by a constricted portion about 4 mm. in diameter. A plug of glass wool is placed above the constriction and the tube is filled with treated zeolite[74] to a height of 10 cm. This requires about 3 g. of the adsorbent. The zeolite filter is fastened to the bottom of the condenser by two 2-inch steel springs, joined to glass hooks. At the bottom of the apparatus is a 2-liter suction flask which acts as a reservoir. This is equipped with a two-way stopcock for connection to suction or to the atmosphere. The receiving tube, calibrated at the 10-ml. mark, is suspended from glass hooks in the stopper of the flask to collect the eluate. The condenser, which acts intermittently as a steam jacket, is connected to a laboratory steam line, or to a 1-liter flask of boiling water equipped with a two-way stopcock.

Pass an aliquot of the filtered extract containing 15–50 μg. of thiamine through the zeolite column at room temperature. Maintain a filtration rate of 3 or 4 drops per second with the aid of mild suction. Then pass steam

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[72] Hochberg, Melnick, and Oser: *Cereal Chem.* 26, 83, (1945).

[73] As much as 20 g. may be taken. Samples rich in protein should be hydrolyzed with 1 g. of pepsin at pH 1-2 after extraction.

[74] Prepare the adsorbent as directed in the thiochrome procedure above

through the outside jacket, and pour 30 ml. of water on the column. Allow to heat for one-half minute, then draw through with full suction to wash and heat the zeolite adsorbate. Elute the thiamine immediately by passing 10 ml. of 25 per cent potassium chloride solution in 0.1 N hydrochloric acid down the wall of the hot condenser. Collect at the rate of approximately 1 drop per 2 seconds, drawing through the final few drops by suction. Wash the zeolite column with 200 ml. of distilled water under full suction and with the steam on. Cool the column by running the last 50 ml. of wash through with the steam turned off. The apparatus is then ready for the next extract.

Pass a standard solution containing 30 μg. of thiamine in 50 ml. of acidulated water at pH 4.5 through the column in the same manner as the test extract.

Colorimetric Procedure. **Transfer the eluate to a 100-ml. centrifuge tube. Add 10 ml. of alcohol-phenol reagent,[75] previously poured into the receiving tube as a wash, then 2 drops of a 1 per cent alcoholic solution of thymol blue. Bring all samples to this point before proceeding. Handling one tube at a time, add 2 N sodium hydroxide dropwise, with constant stirring, till the first distinct blue color is produced, then immediately add 25 ml. of freshly prepared thiamine reagent.[76] Allow to stand at least 2 hours, or preferably overnight, at room temperature. Add 5 to 15 ml. of xylene and shake vigorously for 3 minutes. Centrifuge. Transfer the xylene layer by means of a U-tipped pipet to a Nessler tube, a visual colorimeter cup, or the absorption cell of a photoelectric colorimeter (520 mμ filter), and evaluate versus the standard solution.**

CALCULATION. The color intensity is linear in the range recommended. Calculate the thiamine content of the sample using the formula:

$$\frac{U}{S} \times 30 \times \frac{200}{G} = \mu\text{g. thiamine per g. of sample}$$

U/S is the ratio of the concentration of red pigment in the unknown to that in the standard (the ratio of the photometric densities when a photoelectric colorimeter is employed); G is the weight of the sample in grams.

BIOLOGICAL METHODS

The symptoms of thiamine deficiency which have been adapted to biological assay are polyneuritis and anorexia. Pigeons,[77] chicks,[78] and rats[79] are employed in the methods based on cure or prevention of polyneuritis, which have the advantage of specificity but are not as adaptable to quantitative treatment as are growth methods. Curative methods are based on the duration of the cure of head retraction in birds or of paralytic convulsions in rats, resulting from feeding graded doses of the test material. The difficulty arises in determining precisely when the symptoms have advanced sufficiently to administer the test dose and when "cure" is effected, the question being one of hours rather than days, and subject to considerable personal judgment. The pigeon-curative method has strong defenders, particularly among English workers. Before the differentiation of vitamin B was established, most of the studies on the antineuritic properties of the vitamin B complex were conducted on pigeons, because rats rarely exhibit typical symptoms of polyneuritis even though they cease to grow when deprived of this complex. Polyneuritis can be produced in rats by maintaining a state of partial deficiency in order that the rats survive long enough for the symptoms to appear. A method based on the growth response of rats to graded doses of thiamine has been adopted by the Association of Official Agricultural Chemists.

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[75] Dissolve 3.9 g. of phenol in 500 ml. of 95 per cent alcohol. Store in an amber glass bottle.

[76] Dissolve 0.635 g. of *p*-aminoacetophenone in 9 ml. of concentrated hydrochloric acid and dilute to 100 ml. with distilled water. Dissolve 22.5 g. of sodium nitrite in distilled water and dilute to 500 ml. Dissolve 20 g. of sodium hydroxide and 28.8 g. of sodium bicarbonate in distilled water and dilute to 1000 ml.

Diazonium Salt Solution: Pipet 5 ml. of *p*-aminoacetophenone solution into a 50-ml. cylinder surrounded with chopped ice and water and provided with a stirrer. Add 5 ml. of sodium nitrite solution slowly, and stir for 10 minutes. Add an additional 20 ml. of nitrite solution slowly with stirring and keep in the ice bath for 30 minutes longer. Store at a temperature below 5° C. and use the solution on the same day it is prepared.

Thiamine Reagent: Add 10 ml. of the diazonium salt solution to 137 ml. of sodium hydroxide-bicarbonate solution with vigorous stirring. Allow to stand till the initial pink coloration changes to pale yellow (5–20 minutes), then use immediately.

[77] Kinnersley, Peters, and Reader: *Biochem. J.*, **22**, 276 (1928); Coward, Burn, Ling, and Morgan: *ibid.*, **17**, 1719 (1933); Waterman and Ammerman: *J. Nutrition*, **10**, 161 (1935); Carter and O'Brien: *Biochem. J.*, **31**, 2264 (1937).

[78] Arnold and Elvehjem: *J. Nutrition*, **15**, 403 (1937).

In the complete absence of thiamine, weanling rats show a gain in weight for the first week or so, followed by a rapid decline and death in 25–40 days. If life is prolonged on a submaintenance allowance of thiamine, the characteristic picture of polyneuritis appears, including lack of appetite, torpidity, spastic movements of the head and legs, and, in more severe cases, head retraction and paralysis of the hind legs. Lack of muscular coordination and a tendency to hold the head to one side and walk in circles are frequently manifested. (See Fig. 276, p. 1135.)

After 4–7 weeks, evidences of polyneuritis are seen, including incoordination, spasticity, and rolling movements. Spinning the rat by its tail will usually evoke convulsive seizures. When these become distinctive and consistent, the test foods or extracts are fed or injected in sufficient dosage to effect cure, the quantitative criterion being the minimum dose which will prevent a return of the symptoms for at least five days. This method, as adapted by the U.S. Pharmacopeia, is described in full on p. 1148.

The rat-growth method of Sherman and Chase[80] has been the basis of many evaluations of the vitamin B_1 content of foods and, except for the composition of the basal diet, is substantially the biological assay method for thiamine tentatively adopted by the A.O.A.C. (see below).

Coprophagy must be prevented in assays for the B vitamins since bacterial synthesis in the intestinal tract may produce significant concentrations of vitamins. Thus rats may grow on a B-free diet, this condition being known as "refection."[81] Partial digestion (dextrinization or gelatinization) of the starch in the diet by boiling has been recommended to avoid refection.

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[79] Smith: *U.S. Pub. Health Repts.*, **45**, 116 (1930).

[80] Chase, cited by Sherman and Smith: *The Vitamins*, 2d ed. New York, Chemical Catalog Co. (now Reinhold Publishing Corp.), 1931.

[81] Fridericia, Freudenthal, Gudjonnsson, Johansen, and Schoubye: *J. Hyg.*, **27**, 70 (1927); Roscoe: *J. Hyg.*, **27**, 103 (1927); Kon and Watchorn: *J. Hyg.*, **27**, 321 (1928); Kelly and Parsons: *J. Nutrition*, **13**, 453 (1937).

Rat-Growth Assay for Thiamine:[82] ***Principle.*** Rats, depleted of their thiamine reserves by means of a vitamin B_1-free basal diet, are fed graded supplements of thiamine in the form of the standard or test materials. The relative growth responses to the doses of assay material and of pure thiamine constitute the basis for evaluation.

Procedure.* Healthy rats not exceeding 28 days of age and weighing between 40 and 50 g. are placed in individual cages with raised wire-mesh floors (not less than 8 × 8 mm. mesh). Water and basal diet are fed *ad libitum.

BASAL THIAMINE-DEFICIENT DIETS

Sherman and Chase		*A.O.A.C.*	
Casein (thiamine-free)[83]	18	Casein (thiamine-free)[83]	18
Starch[84]	53	Sucrose	60
Salt mixture[85]	4	Salt mixture[85]	4
Butterfat[86]	8	Liver extract	1
Codliver oil[86]	2	Codliver oil	2
Autoclaved baker's yeast[87]	15	Autoclaved yeast[87]	5
		Autoclaved peanuts[88]	10
		Pyridoxine	0.0002

The rats are weighed at intervals not exceeding 3 days. After 10 (but before 30) days the rats are depleted, as evidenced by stationary or declining weight over any 5-day period. They are then assembled into groups of 8 or more, one group being maintained on the basal diet as the negative control, the remainder receiving either graded doses of U.S.P. thiamine hydrochloride (0.1 ml. = 3.0 μg. thiamine) fed directly into the mouth by means of a syringe with a blunt-edged 18-gauge needle, or measured doses of the assay material corresponding in expected thiamine content to the amounts of standard fed to the reference groups. The intervals between doses should be constant for both reference and assay groups; each dose is computed by multiplying the next lower dose by a constant factor (e.g., 1.5 or 2).

CALCULATION. During the 4-week assay period, weights are recorded at weekly intervals. The average net gains of the reference groups are plotted and the average gains of the assay groups interpolated on this curve in terms of thiamine content for each dose level of the assay material.

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[82] Cited by Sherman and Smith: *The Vitamins*, 2d ed. New York, Reinhold Publishing Corp., 1931; Association of Official Agricultural Chemists: *Official and Tentative Methods of Analysis of the Association*, 7th ed. Washington, D.C., 1950.

[83] Thiamine-free casein may be prepared as follows: Stir 400 g. of casein with 2 liters of 60 per cent (by weight) alcohol for ½ hour, let stand 5½ hours, filter with suction, and wash with 1 liter of 60 per cent alcohol. Repeat, letting stand 18 hours and adding a final wash with 1 liter of 90 per cent (by weight) alcohol. Spread on trays to dry in air. Vitamin-free casein is supplied by the Borden Co., Bainbridge, N. Y., and by General Biochemicals, Chagrin Falls, Ohio.

[84] See p. 1146.

[85] See U.S.P. salt mixture, p. 1262.

[86] In place of butterfat and codliver oil, 10 per cent hydrogenated vegetable oil may be used together with 2000 U.S.P. units of vitamin A and 200 units of vitamin D per 100 g. of basal diet added in the form of high-potency fish liver oil or concentrate.

[87] See footnote 93, p. 1150.

[88] See footnote 94, p. 1150.

If desired the data may be treated statistically according to the analysis of variance illustrated for vitamin A in Appendix VI.

Results are preferably expressed in gravimetric units—e.g., mg. of thiamine per 100 g. of assay material; the International or U.S. Pharmacopeial unit is the potency of 0.003 mg. (3 μg.) of crystalline thiamine hydrochloride.

ASSAYS FOR THIAMINE[89]

Biological Method of the U.S. Pharmacopeia XIV.

The biological assay, comprising the recording of observations of rats throughout specified periods of their lives, while being maintained on specified dietary regimens, and the interpretation of such data, is as follows:

PRELIMINARY PERIOD. Throughout the preliminary period each rat shall be raised under the immediate supervision of, or according to directions specified by, the assayer. Throughout the preliminary period the rats shall be maintained on a dietary regimen which shall provide for normal development in all respects, except that the thiamine hydrochloride intake may be restricted.

DEPLETION PERIOD. A rat shall be suitable for the depletion period when the age of the rat does not exceed 30 days, and if the body weight of the rat does not exceed 50 g. and if the animal manifests no evidence of injury or disease or anatomical abnormality which might hinder growth and development. Throughout the depletion period each rat shall be provided with the thiamine hydrochloride test diet and water (U.S.P.) *ad libitum*, and during this period no other dietary supplement shall be available to the animal. Throughout the depletion period and until the assay shall have been completed, the rats shall be kept in cages provided with a wire cloth bottom, each mesh of which shall be not less than 8 mm.

ASSAY PERIOD. A rat shall be suitable for the assay period, provided that the depletion period shall not have exceeded 75 days, and provided that the rat shall manifest evidence of thiamine hydrochloride deficiency characterized by acute polyneuritis. Throughout the assay period each rat shall be kept in an individual cage and provided with the thiamine hydrochloride test diet, compounded from the same lots of ingredients, and water (U.S.P.) *ad libitum.* On the day beginning the assay period there shall be administered to each rat a single dose of the reference standard of such size that it will produce in individual animals a curative period of not less than 5 days and not more than 15 days. All of the rats used in any one assay shall receive the same quantity of the reference standard. Each rat shall then be observed for the cure of and recurrence of polyneuritis, and when polyneuritis reaches the same acute stage observed when the reference standard was administered, a single dose of the assay product shall be administered. The animals shall then be observed to determine if polyneuritis is cured, and, if so, observation shall be made of the duration of the period. Each assay shall include successive administration of the reference standard and assay product to not less than eight rats. The assay product may be administered orally or parenterally, but in any one assay the reference standard shall be administered in the same manner as the assay product, and the quantity of the assay product administered to each rat shall be the same.

RECORDING OF DATA. On the day beginning the depletion period and at intervals of not more than 7 days during the depletion period, a record shall be made of the body weight of each rat. On about the twenty-fifth day and each day thereafter for the remainder of the depletion period, each rat shall be observed for symptoms of polyneuritis. The following dates shall be recorded:

1. The day on which the reference standard is administered.

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[89] Grateful acknowledgment for permission to reproduce these methods (U.S.P. XIV) is made to Dr. Lloyd C. Miller and the Board of Trustees of the U.S. Pharmacopeial Convention, Inc.

2. The day on which cure of polyneuritis is observed following the administration of the reference standard.

3. The day on which acute polyneuritis recurs and the assay product is fed.

4. The day on which cure of polyneuritis is observed following the administration of the assay product.

5. The day on which acute polyneuritis recurs after the administration of the assay product.

THIAMINE HYDROCHLORIDE OR VITAMIN B_1 POTENCY OF THE ASSAY PRODUCT. In determining the thiamine hydrochloride potency of the assay product, the duration of the curative period following the administration of the reference standard and the assay product shall be considered. The dose of the assay product administered contains an amount of the thiamine hydrochloride equal to or greater than that contained in the dose of the reference standard administered if that quantity promotes in the assay animals a total curative period (the sum of the number of days of the curative period of each of the animals) equal to or greater than the total curative period produced by administration of the reference standard.

DEFINITIONS. As used herein, unless the context otherwise indicates, the term *acute polyneuritis* means that stage of thiamine hydrochloride deficiency in which the animal regains control of the voluntary muscles, as evidenced by standing or walking, a few seconds after extreme muscular contraction, which has been induced by twirling the rat by its tail (the onset of acute polyneuritis is invariably accompanied by loss in body weight). The term *assay period* means the interval in the life of a rat between the last day of the depletion period and the final observation following the administration of the assay product; the term *assay product* means a product under examination for its thiamine hydrochloride potency; the term *curative period* is the interval of time between the administration of thiamine hydrochloride and the subsequent recurrence of acute polyneuritis after a complete disappearance of polyneuritis symptoms, and the duration of the curative period is expressed as the number of days in that interval; the term *cure of polyneuritis* means the complete disappearance of polyneuritic symptoms and is invariably accompanied by increase in body weight; the term *depletion period* means the interval in the life of a rat during which its food intake is only the thiamine hydrochloride test diet and water (U.S.P.); the term *preliminary period* means the interval in the life of a rat prior to the depletion period; the term *reference standard* means the U.S.P. Thiamine Hydrochloride Reference Standard;[90] the term *thiamine hydrochloride test diet* means a uniform mixture, which has not been compounded for more than 7 days, of the following food materials and in the proportions designated:

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[90] *Suggestions for Using the U.S.P. Thiamine Hydrochloride Reference Standard.*

Before preparing a solution of the Reference Standard, dry it to constant weight in a desiccator over phosphorus pentoxide.

Precautions to be Taken in the Preparation of Solutions. Because of the hygroscopic nature of the completely desiccated U.S.P. Thiamine Hydrochloride Reference Standard, it is preferable to transfer the quantity required for a test to a small glass-stoppered weighing bottle, in which it can then be weighed on a microbalance, or an ordinary balance according to the number of tests for which it is to be used. Even without such precautions, however, exposure to air during weighing will not cause an increase in weight of more than about 0.6 per cent, if the operations are completed within 5 minutes.

Neutral and alkaline solutions of thiamine hydrochloride are unstable, and water-acid solutions are readily infected by molds, which inactivate the vitamin. Therefore, stock solutions should be prepared using 25 per cent alcohol and containing sufficient hydrochloric acid to make the solution approximately 0.002 N. A convenient strength for a stock solution is 0.5 mg. of thiamine hydrochloride to each ml. These solutions are stable if stored at about 4°.

Solutions of suitable strength for animal dosage (20 to 100 μg. per ml.) must be made at least twice weekly from the stock solution by dilution with water. Such dilutions must be kept at a low temperature and examined daily for mold.

Thiamine Hydrochloride or Vitamin B_1 Test Diet

Sucrose	60.25 per cent
Casein[91]	18 per cent
Salt Mixture[92]	4 per cent
Autoclaved Yeast[93]	5 per cent
Autoclaved Peanuts[94]	10 per cent
Purified Liver Extract[95]	0.75 per cent
Cod Liver Oil	2 per cent

The Liver Extract used in this *Test Diet* must contain, in each g., at least that amount of material from liver which, when given daily to patients with pernicious anemia, has produced a satisfactory hematopoietic response.

Chemical (Thiochrome) Method of the U.S. Pharmacopeia.

DOUBLE-NORMAL SODIUM ACETATE. Dissolve 275 g. of reagent sodium acetate in sufficient water to make 1000 ml.

BROMOCRESOL GREEN pH INDICATOR. See p. 39.

THYMOL BLUE pH INDICATOR. See p. 39.

ENZYME SOLUTION. Prepare on the day on which it is to be used a 10 per cent solution in water of an enzyme preparation potent in diastatic and phosphatatic activity.

BASE EXCHANGE SILICATE. Purify an artificially prepared silicate of the base-exchange type, in the form of a granular powder of 50- to 80-mesh size, in the following manner: Place a convenient quantity (100 to 500 g.) of the base exchange silicate in a suitable beaker, add sufficient hot 3 per cent acetic acid ($HC_2H_3O_2$) to cover the material, and boil for 10 to 15 minutes, stirring frequently. Allow to settle, and decant the supernatant liquid. Repeat this washing three times. Then wash in a similar manner three times with a hot 25 per cent solution of potassium chloride (1 part by weight of KCl in 4 volumes of solution) and finally wash with boiling water until the last washing gives no reaction for chloride. Dry the material at approximately 100° and store in a well-closed container.

BASE EXCHANGE TUBE. The base exchange glass tube has an over-all length of 200 mm. A reservoir at the upper end is 50 mm. in length and 25 mm. in diameter. This converges into the adsorption tube which is 5 to 6 mm. in internal diameter and approximately 140 mm. long. At the lower end the tube is drawn into a capillary approximately 10 mm. long and of such diameter that when the tube is charged the rate of flow will be not more than 1 ml. per minute.

Prepare the tube for use by placing over the upper end of the capillary, with the aid of a glass rod, a pledget of fine glass wool. Add a water suspension of 1.0 to 2.0 g. of the purified base exchange silicate to the adsorption tube, taking care to wash down all of the silicate from the walls of the reservoir. To keep air out of the adsorption column, a layer of liquid must be kept above the surface of the silicate throughout the adsorption process and the tube may be prevented from draining by placing a rubber cap (filled with water to avoid inclusion of air) over the lower end of the capillary.

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[91] The casein shall be free from demonstrable traces of thiamine hydrochloride. (See footnote 83, p. 1147.)

[92] The salt mixture shall be the salt mixture described on p. 1262, or a salt mixture having essentially the same proportions of the elements.

[93] Dried yeast which has been autoclaved in steam at 15 pounds pressure for 5 hours with the yeast spread in a layer not more than 6 mm. in depth and then dried at a temperature not exceeding 65°.

[94] Unroasted shelled No. 1 grade Virginia peanuts are crushed in a food chopper, autoclaved in steam at 15 pounds pressure for 5 hours with the ground peanuts spread in a layer not more than 12 mm. in depth, and then dried at a temperature not exceeding 65°. This preparation may be incorporated in the basal diet by grinding with the requisite quantity of sucrose.

[95] Dissolve 100 g. of Liver Extract in 1 liter of 0.6 per cent sodium bisulfite solution. Let stand 24 hours in a well-stoppered bottle; acidify with hydrochloric acid to a pH of 1.5. Concentrate by distillation under reduced pressure at a temperature not exceeding 50° to one-half the original volume. Dry on vitamin B_1-free casein at a temperature not exceeding 65°.

NEUTRAL POTASSIUM CHLORIDE SOLUTION. Dissolve 250 g. of reagent potassium chloride in sufficient water to make 1000 ml.

ACID POTASSIUM CHLORIDE SOLUTION. Add 8.5 ml. of reagent hydrochloric acid to 1000 ml. of the neutral potassium chloride solution.

SODIUM HYDROXIDE SOLUTION, 15 PER CENT. Dissolve 15 g. of sodium hydroxide in sufficient water to make 100 ml.

POTASSIUM FERRICYANIDE SOLUTION, 1 PER CENT. Dissolve 1 g. of reagent potassium ferricyanide in sufficient water to make 100 ml. This solution must be freshly prepared on the day it is used.

OXIDIZING REAGENT. Prepare the solution by mixing 4.0 ml. of the 1 per cent potassium ferricyanide solution with sufficient of the 15 per cent sodium hydroxide solution to make 100 ml. This solution should be used within 4 hours.

QUININE SULFATE STOCK SOLUTION. Quinine sulfate solution is used to govern the reproducibility of the fluorophotometer. A stock solution of quinine sulfate is prepared by dissolving 10 mg. of quinine sulfate in sufficient 0.1 N sulfuric acid to make 1000 ml. Preserve this solution in light-resistant containers.

QUININE SULFATE STANDARD SOLUTION. Dilute 1 volume of the quinine sulfate stock solution with 39 volumes of 0.1 N sulfuric acid. This solution fluoresces to approximately the same degree as the thiochrome obtained from 1 μg. of thiamine hydrochloride. Preserve this solution in light-resistant containers.

THIAMINE HYDROCHLORIDE STOCK SOLUTION. Weigh accurately not less than 25 mg. of U.S.P. Thiamine Hydrochloride Reference Standard which has been kept in a desiccator over phosphorus pentoxide for at least 16 hours. Since the Reference Standard is hygroscopic, precautions must be taken to avoid absorption of moisture. Dissolve in 20 per cent alcohol adjusted to a pH of 3.5 to 4.3 with hydrochloric acid and make up to a volume of 1000 ml. Store in a cool place in a well-closed, light-resistant container.

THIAMINE HYDROCHLORIDE STANDARD SOLUTION. From a portion of the stock solution that has been warmed to room temperature, transfer to a 100-ml. volumetric flask an aliquot containing exactly 100 μg. of thiamine hydrochloride, and dilute to 100 ml. with water adjusted to a pH of 3.5 to 4.3 with hydrochloric acid. Each ml. of this solution contains 1 μg. of thiamine hydrochloride.

Dilutions of this solution are treated in the same manner as that used in the *Preparation of Assay Solution* with respect to acid digestion, enzyme treatment, adsorption, and elution from the base exchange silicate.

PREPARATION OF ASSAY SOLUTION. The amount of material taken for the assay should be such that the ratio of the volume of 0.1 N sulfuric acid used for the extraction to the quantity of sample is at least 15 to 1, and the content of thiamine equivalent to 30 to 100 μg. of thiamine hydrochloride. Place the accurately weighed quantity of the material to be assayed in 65 ml. of 0.1 N sulfuric acid contained in a 100-ml. centrifuge tube and digest it on a steam bath, with frequent mixing, for 30 minutes, or heat in an autoclave at 121° to 123° for 15 minutes. The liquid must remain acid during the digestion. If it is not distinctly acid to the thymol blue pH indicator, add sufficient diluted sulfuric acid to make it acid. Cool, and adjust the pH to between 4 and 4.5 by the addition of 2 N sodium acetate solution, using bromocresol green pH indicator as an external indicator in conjunction with a spot plate. Add 5 ml. of the enzyme solution, mix, and incubate at 45° to 50° for 3 hours. Cool, centrifuge the mixture until the supernatant liquid is clear or practically so, and quantitatively transfer the supernatant liquid to a 100-ml. volumetric flask. Wash the residue by centrifuging with 10 ml., then with 5 ml. of 0.1 N sulfuric acid. Add the washings to the supernatant liquid and dilute to 100 ml. with water.

Pass through the prepared base exchange tube an aliquot of the solution estimated to contain 5 to 10 μg. of thiamine, wash the tube with three 5-ml. portions of boiling water, taking care to prevent the surface of the liquid from falling below the surface of the silicate.

Elute the thiamine from the *base exchange silicate* by passing successively through the tube small portions of hot *acid potassium chloride solution.* Collect the first 15 to 20 ml. of the liquid (*eluate*) in a glass-stoppered, 25-ml. volumetric flask, cool, and dilute to a volume of 25 ml. with acid potassium chloride solution. *This constitutes the assay solution.*

Oxidation of Thiamine to Thiochrome and Measurement of Fluorescence. Determine the thiamine content of the oxidized assay solution by comparing the intensity of fluorescence of an extract of this solution exposed to ultraviolet rays ranging from 350 mμ to 400 mμ with that of oxidized Thiamine Hydrochloride Standard Solution. The intensity of the fluorescence is proportional to the quantity of thiamine present and may be measured with the aid of various instruments.

To oxidize thiamine to thiochrome, add to quantities of the assay solution and of the similarly treated Thiamine Hydrochloride Standard Solution containing from 0.10 to 2 μg. of thiamine, sufficient acid potassium chloride solution to produce a volume of 5 ml. Then add, with mixing, 3 ml. of oxidizing solution. Within 2 minutes add 13 ml. of isobutyl alcohol and shake vigorously for at least 1½ minutes. Centrifuge the mixture at a low speed until a clear supernatant solution is obtained. Measure in a fluorometer the intensity of fluorescence of the isobutyl alcohol solution directly if clear, or, if cloudy, after shaking with 2 g. of anhydrous sodium sulfate. Compare with this the intensity of fluorescence produced after oxidation of the properly prepared Thiamine Hydrochloride Standard Solution. Quinine Sulfate Standard Solution is used to govern the reproducibility of the instrument. Correction must be made for fluorescence produced by substances other than thiamine by determining the intensity of fluorescence of Thiamine Hydrochloride Standard Solution, and assay solutions treated as described above, but with 15 per cent sodium hydroxide solution replacing the oxidizing reagent.

The _Thiochrome Method_ is applicable to substances such as Thiamine Hydrochloride, and a number of other products, but cannot be relied upon when certain interfering substances are present; in the latter case, use the _Biological Method,_ p. 1148.

RIBOFLAVIN

Riboflavin owes its discovery as one of the components of the B vitamins to its heat-stability and its characteristic fluorescence. The growth-promoting effect of certain water-soluble yellow dyes extracted from foods was noted by several investigators early in 1933. The biological activity was proportional not only to the intensity of the yellow color, but also to the fluorescence of the extracts.[96] Kuhn, György, and Wagner-Jauregg[97] isolated crystalline riboflavin from several foods and demonstrated the growth-promoting properties of the vitamin on rats. Riboflavin was synthesized shortly thereafter.[98]

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[96] Booher: *J. Biol. Chem.*, **102**, 39 (1933).

[97] Kuhn, György, and Wagner-Jauregg: *Ber.*, **66B**, 317, 576, 1034 (1933).

The clinical syndrome of ariboflavinosis was first described in 1938 by Sebrell and Butler.[99] Ariboflavinosis occurs more frequently among infants and children[100] than among adults and since its prevention depends upon an adequate diet it occurs most often among low income groups.[101] It is one of the more common dietary deficiency diseases and is usually associated with lack of other vitamins, notably niacin. It is characterized by the occurrence of cheilosis and glossitis.

The naturally occurring flavins—lactoflavin, ovoflavin, hepatoflavin, and verdoflavin—isolated respectively from milk, eggs, liver, and grass, are all chemically identical with riboflavin.

Physiological and Clinical Aspects of Riboflavin. Vitamin B_2 deficiency affects primarily the ectodermal tissues, producing lesions of the skin, eye, and nervous system. One of the earliest symptoms is cheilosis, manifested at first by transverse fissures at the corners of the mouth, raw and scaly lips, and finally by many vertical, deep fissures. The tongue assumes a purplish or magenta tinge and glossitis (flattening of the papillae) is observed. A seborrheic dermatitis occurs at the body folds; e.g., at the *alae nasi*, and in the scrotal and vulvar regions. The ocular manifestations of riboflavin deficiency include dryness, burning and itching, photophobia and lacrimation, and vascular invasion particularly at the scleral junction of the cornea. Cataracts due to pigmentation and capillary invasion of the cornea are known to occur in animals on a riboflavin-deficient diet, but it is uncertain to what extent human cataracts result from dietary causes. Though these symptoms may not individually be specific, cheilosis, glossitis, seborrheic dermatitis, and corneal vascularization constitute a group of signs which separately or in combination are observed in ariboflavinosis. The symptomatology of clinical riboflavin deficiency is outlined in the syllabus on p. 1291.

Clinical tests of the riboflavin content of blood and urine have been employed for the diagnosis of riboflavin deficiency. Normal individuals have a blood level of 0.5 μg. per ml. and when subsisting on an adequate diet excrete 500 to 800 μg. per day in the urine. The excretion and blood levels show diurnal variations depending upon the dietary intake. A diagnostic procedure involves measurement of the urinary excretion for 4 hours following the intravenous injection of 1 mg. of riboflavin.[102] Blood riboflavin may be determined by the microbiological procedure, employing *Lactobacillus casei*, or by the fluorometric method. In addition to riboflavin, the latter measures uroflavin, a metabolite of the vitamin.

Riboflavin is required by all animals and many microorganisms. A deficiency of the vitamin in young animals results in inhibition of growth terminated by death. In rats, the syndrome includes early atrophy of the testes, involution of the thymus, alopecia, cataract, and degeneration of the main peripheral nerve trunks. In fowls, riboflavin is essential for normal egg production and hatchability. Peripheral nerve degeneration in chicks is responsible for "curled toe paralysis." In monkeys, riboflavin deficiency results in anemia and leukopenia.

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[98] Kuhn, Reinemund, Weygand, and Ströbele: *Ber.*, **68**, 1765 (1935), Karrer and associates: *Helv. chim. acta*, **18**, 1435 (1935).

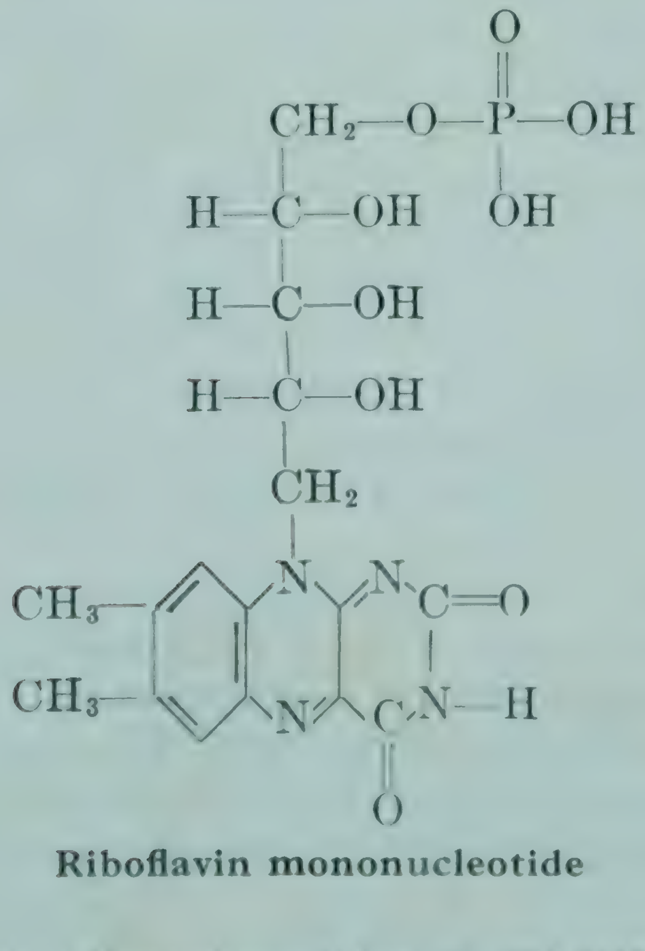
[99] Sebrell and Butler: *Pub. Health Rep.*, **53**, 2282 (1938); **54**, 2121 (1939).

[100] Spies, Bean, Vilter, and Huff: *Am. J. Med. Sci.*, **200**, 697 (1940).

[101] Goldsmith: *Southern Med. J.*, **36**, 108 (1943).

[102] Najjar and Holt: *Bull. Johns Hopkins Hosp.*, **69**, 476 (1941).

Riboflavin plays an important role in many enzyme systems. In 1932, Warburg and Christian isolated a yellow respiratory enzyme from yeast.[103] This enzyme was shown to consist of a combination of riboflavin phosphate and a protein. The riboflavin phosphate, a mononucleotide, could be separated from the protein (apoenzyme) by dialysis against a weak acid. Neither fraction alone possessed enzymic activity, but the two could be recombined in neutral solution to produce the original enzyme. The yellow enzyme of Warburg and Christian can participate in a series of enzyme reactions involved in the metabolism of carbohydrates. It is capable of transporting hydrogen from reduced coenzyme II, a niacin coenzyme which attacks hexosemonophosphate, for example, regenerating that enzyme of first attack. The reduced yellow enzyme may be reoxidized itself by molecular oxygen. This series of reactions, however, is extremely slow and is probably of no physiological significance. Two other enzymes, cytochrome C reductase[104] and L-amino acid oxidase,[105] contain riboflavin phosphate. The former transports hydrogen from reduced coenzyme II to cytochrome C at a rate which is sufficiently rapid to be physiologically important. This suggests that riboflavin functions in the oxidation-reduction metabolism of plant and animal tissues. L-Amino acid oxidase catalyzes the oxidation of L-amino acids and of α-hydroxy acids.



Riboflavin mononucleotide

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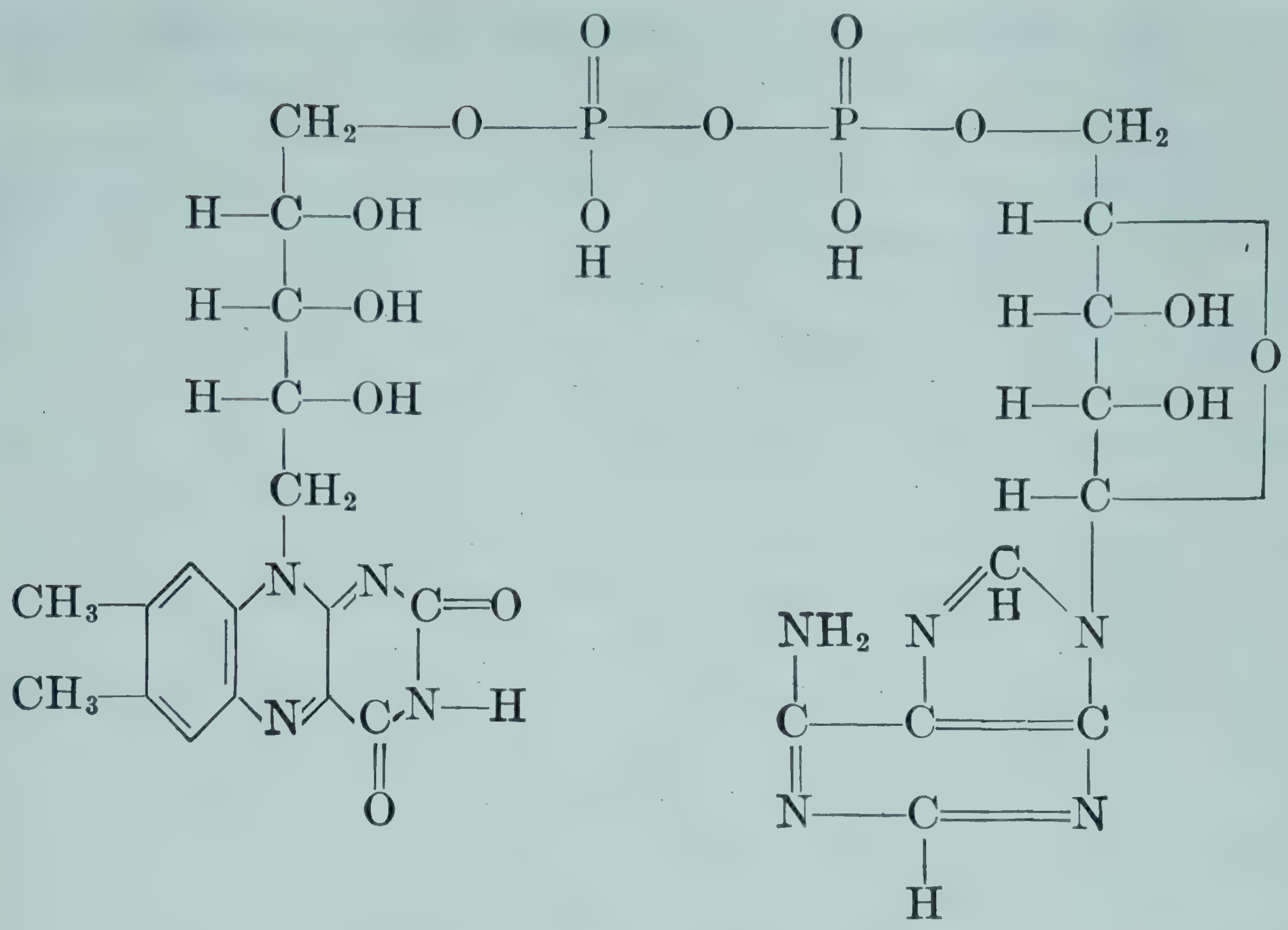
[103] Warburg and Christian: *Naturwissenschaften*, **20**, 980 (1932); *Biochem. Z.*, **254**, 438 (1932); **258**, 496 (1933). Theorell: *Biochem. Z.*, **272**, 155 (1934); **275**, 37 (1934); **278**, 263 (1935).

[104] Haas, Harrer, and Hogness: *J. Biol. Chem.*, **143**, 344 (1942). Haas, Horecker, and Hogness: *ibid.*, **130**, 425 (1939).

[105] Blanchard, Green, Nocito, and Ratner: *J. Biol. Chem.*, **161**, 583 (1945).



Riboflavin-adenine dinucleotide

Riboflavin also participates in enzyme reactions as a dinucleotide prosthetic group, consisting of riboflavin, two phosphoric acids, ribose, and adenine. This coenzyme is found in xanthine oxidase, diaphorase, D-amino acid oxidase, a synthetic enzyme of Warburg and Christian, fumaric hydrogenase, liver aldehyde oxidase, and the Haas enzyme. Xanthine oxidase[106] (Schardinger enzyme, aldehydrase) is found in liver and milk and catalyzes the oxidation of aliphatic and aromatic aldehydes, reduced coenzyme I (another niacin coenzyme) and of a number of purines (including xanthine and hypoxanthine). In the presence of air, the reduced enzyme transfers its hydrogen to oxygen, forming hydrogen peroxide which inhibits further action. The accumulation of hydrogen peroxide is prevented by the presence of catalase, an iron-porphyrin-protein enzyme which catalyzes the decomposition of hydrogen peroxide to water and oxygen. The hydrogens of xanthine oxidase, like those of numerous other reduced enzymes, can be accepted also by methylene blue and by other oxidizing dyes. Two different diaphorases[107] have been found in plants, animal tissues, and microorganisms. In the presence of suitable proteins, one of these oxidizes reduced coenzyme I and reduced coenzyme II. D-Amino acid oxidase[108] found in animal tissues, notably kidney and liver, converts D-α-amino acids to α-keto acids. Different D-amino acids are acted upon at different rates by this enzyme. Fumaric hydrogenase, another enzyme which contains riboflavin dinucleotide, catalyzes the reduction of fumaric acid in the presence of certain reduced dyes[109] to succinic acid. Aliphatic and aromatic aldehydes are oxidized by liver aldehyde oxidase,[110] a riboflavin dinucleotide enzyme found in the liver of mammals.

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[106] Schardinger: *Z. Untersuch. Nahr. u. Genussm.*, **5**, 22 (1902).

[107] Dewan and Green: *Biochem. J.*, **31**, 1069 (1937). Von Euler and Hellström: *Z. physiol. Chem.*, **252**, 31 (1938).

[108] Krebs: *Z. physiol. Chem.*, **217**, 191 (1933); *Klin. Wochschr.*, **11**, 1744 (1932); *Biochem. J.*, **29**, 1620 (1935).

[109] Fischer and Eysenbach: *Ann. Chem.*, **530**, 99 (1937).

The riboflavin enzymes, called flavoproteins, may be dissociated more or less easily and the original enzyme reconstituted by recombining the mono- or dinucleotide with the original protein. The synthetic enzyme of Warburg and Christian[111] consists of the dinucleotide added to the protein of the old yellow respiratory enzyme. The new enzyme differs from the natural enzyme obtained from yeast only in that it contains the dinucleotide in place of the mononucleotide as its prosthetic group; it has the same properties as the old yellow respiratory enzyme. The Haas enzyme consists of the dinucleotide plus a protein from yeast and has the same activity as the old yellow enzyme.

Free riboflavin is phosphorylated in the intestines of higher animals, probably by an enzymatic reaction in which a secretion of the adrenals plays an important part. When iodoacetic acid (an inhibitor of many enzymatic reactions) is administered, or when the adrenals are removed, phosphorylation does not take place and the vitamin is not absorbed from the intestinal tract. Under these conditions, riboflavin is unable to support growth, whereas the phosphate is effective. Human blood cells are capable of synthesizing the dinucleotide from free riboflavin both *in vivo* and *in vitro*.[112]

Since flavoproteins are composed of both riboflavin nucleotides and specific proteins, disturbances in metabolism may result from diminished levels of either constituent. Deficiency of dietary riboflavin results in a decreased concentration of D-amino acid oxidase in liver and kidney[113] and of xanthine oxidase in liver.[114] Both of these conditions may be prevented or improved by feeding riboflavin. The D-amino acid oxidase activity of liver from animals on diets low in riboflavin may be restored by the *in vitro* addition of the nucleotide to the excised tissue.[115] On the other hand, rats on a low-protein diet do not retain dietary riboflavin in the liver, a condition which may be prevented by the administration of methionine. Likewise, liver slices from riboflavin-deficient rats are unable to inactivate estradiol; this ability is retained when methionine is fed. These observations illustrate the interrelationship of riboflavin with protein metabolism and with endocrine function.

Riboflavin is concerned with the regulatory function of the hormones involved in carbohydrate metabolism. The administration of thyroxine to rats causes the loss of liver glycogen unless riboflavin and thiamine are fed simultaneously. The administration of insulin is effective in depancreatized dogs only when these two vitamins are fed. Riboflavin has been employed successfully in the treatment of dark adaptation in certain cases where vitamin A was ineffective. The retina contains free riboflavin which is converted by light to a compound which is involved in stimulation of the optic nerve.

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[110] Gordon, Green, and Subrahmanyan: *Biochem. J.*, **34**, 764 (1940).

[111] Warburg and Christian: *Biochem. Z.*, **298**, 368 (1938).

[112] Klein and Kohn: *J. Biol. Chem.*, **136**, 177 (1941).

[113] Axelrod, Sober, and Elvehjem: *J. Biol. Chem.*, **134**, 749 (1940).

[114] Axelrod and Elvehjem: *J. Biol. Chem.*, **140**, 725 (1941).

[115] Rossiter: *J. Biol. Chem.*, **135**, 431 (1940).

Riboflavin and its two nucleotides are the only naturally occurring compounds which have vitamin B_2 activity. However, several synthetic compounds have been prepared having approximately one-half of the activity of riboflavin. These vitamers lack a methyl group in the six or seven position, or have this group substituted by an ethyl group. Removal of both methyl groups results in the formation of a highly toxic compound. Substitution of an alkyl group in the three position destroys the vitamin activity of riboflavin.

The minimum requirement of riboflavin necessary to maintain tissue stores in man has been estimated as approximately 0.5 mg. per 1000 calories.[116] The recommended dietary allowances of the Food and Nutrition Board of the National Research Council are about 0.6 to 0.7 mg. per 1000 calories as indicated in the table on p. 1108. These allowances are more liberal than minimal requirements in order to provide a margin of safety. Under normal conditions, with an adequate dietary supply of riboflavin, humans excrete approximately one-third of their intake. When larger amounts are ingested, approximately half is excreted in the urine. Very large doses of riboflavin have been fed to dogs and rats with no evidence of toxicity.

Of interest is the relation between riboflavin feeding and experimentally induced neoplasms. Ingestion by rats of 2-acetylaminofluorene, a carcinogen, has been shown to induce ariboflavinosis;[117] high intake of riboflavin (2 mg. per 100 g. diet) inhibited the appearance of choline-induced tumors.[118]

Storage and Synthesis of Riboflavin. Riboflavin is not stored to any considerable extent in animal organs, though higher concentrations are found in the liver and kidney than in other tissues. In plants the younger parts are richer than the older. Broccoli leaves contain twice as much riboflavin as the flower buds; the latter contain more than the stems. Ungerminated seeds, other than peas, contain little riboflavin but appreciable amounts are formed during germination.

Riboflavin is synthesized by most higher plants, by yeasts, and by some bacteria. Though higher animals are unable to synthesize the vitamin themselves, it is produced to a variable extent by microorganisms in the intestinal tract. In the rat this process is influenced by the nature of the carbohydrate in the diet. The ingestion of dextrin and corn starch favors synthesis, whereas sucrose is ineffective. In ruminants, the contribution of riboflavin by bacteria in the rumen is so great that a dietary source of riboflavin is not necessary.[119]

Riboflavin is synthesized commercially and the product is employed on a large scale in bread and flour enrichment and for pharmaceutical purposes. Natural concentrates prepared from whey, yeast, and the anaerobic bacterial fermentation of distillers' slops are employed in animal feeds.

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[116] Williams, Mason, Cusick, and Wilder: *J. Nutrition*, **25**, 361 (1943).

[117] Wase and Allison: *Proc. Soc. Exp. Biol. Med.*, **73**, 147 (1950).

[118] Schaefer, Copeland, Salmon, and Hale: *Cancer Research*, **10**, 786 (1950).

[119] McElroy and Goss: *J. Nutrition*, **20**, 527 (1940).

Distribution of Riboflavin.[120] Riboflavin is widely distributed in plant and animal tissues as free riboflavin, as the phosphate, or as the adenine-dinucleotide phosphate, the latter accounting for 70–90 per cent of the total riboflavin in tissues.[121] The free vitamin is found, for example, in milk, urine, and retina. Riboflavin nucleotides occur more or less firmly bound to proteins. Excellent dietary sources of riboflavin are heart, liver, kidney, muscle, eggs, milk, green leafy vegetables, yeast, and whole grain. The riboflavin content of the average American diet before and after large-scale enrichment of bread and flour was practiced was 1.4 and 1.6 mg. per day, respectively.

Chemistry of Riboflavin. Riboflavin is 6,7-dimethyl-9-(D-1′-ribityl) isoalloxazine. It crystallizes in orange-yellow needles which melt at 282° C. with decomposition. Riboflavin is insoluble in acetone, benzene, chloroform, and ether, and only slightly soluble in water (12 mg. per 100 ml. at room temperature), but very soluble in alkaline solution. Derivatives more soluble in water, such as the monosuccinate, borate, phosphate, and acetate, have been prepared for pharmaceutical use. The vitamin is adsorbed by fuller's earth in acid solution and by frankonit in neutral solution but not by talc, aluminum oxide, calcium carbonate, kaolin, or kieselguhr; it is eluted by 80 per cent acetone or by alkalies such as pyridine-methanol-water mixtures or ammonia water.

Riboflavin forms a yellow solution having a yellow-green fluorescence. It has a characteristic absorption spectrum in the ultraviolet and visible regions, with absorption maxima at 225, 269, 372, and 445 mμ. When exposed to ultraviolet radiation, it shows a fluorescence having a maximum at 565 mμ at pH 6. In acid or alkaline solutions (below pH 3 or above pH 9) the fluorescence of riboflavin increases considerably. Riboflavin is amphoteric, having an isoelectric point at pH 6. It is optically active; $[\alpha]_D^{20} = -114°$ in 0.1 N sodium hydroxide, this value decreasing in neutral or acid solution. Riboflavin is stable to heat in neutral and acid but not in alkaline solutions. It is also unstable to visible and ultraviolet radiation, especially at alkaline pH and at elevated temperatures. The riboflavin content of milk in clear bottles or of bread in transparent wrappings may drop significantly after only a few hours' exposure to sunlight. Alkaline irradiation of riboflavin produces lumiflavin (6,7,9-trimethylisoalloxazine), a fluorescent degradation product soluble in chloroform. In neutral or acid solution, lumichrome (6,7-dimethylalloxazine) is formed. Riboflavin is easily reduced by hydrogen in the presence of a catalyst, by zinc in acid, by sodium hydrosulfite, by hydrogen sulfide in alkaline solution, and by other reducing agents. Reduction is accompanied by a shifting of bonds in the isoalloxazine ring as shown on p. 1159. Leucoriboflavin, the reduction product, is a colorless nonfluorescent compound which is easily reoxidized by shaking with air in solution. The oxidation-reduction reaction is probably of great importance in the function of riboflavin nucleotide in cellular respiration.

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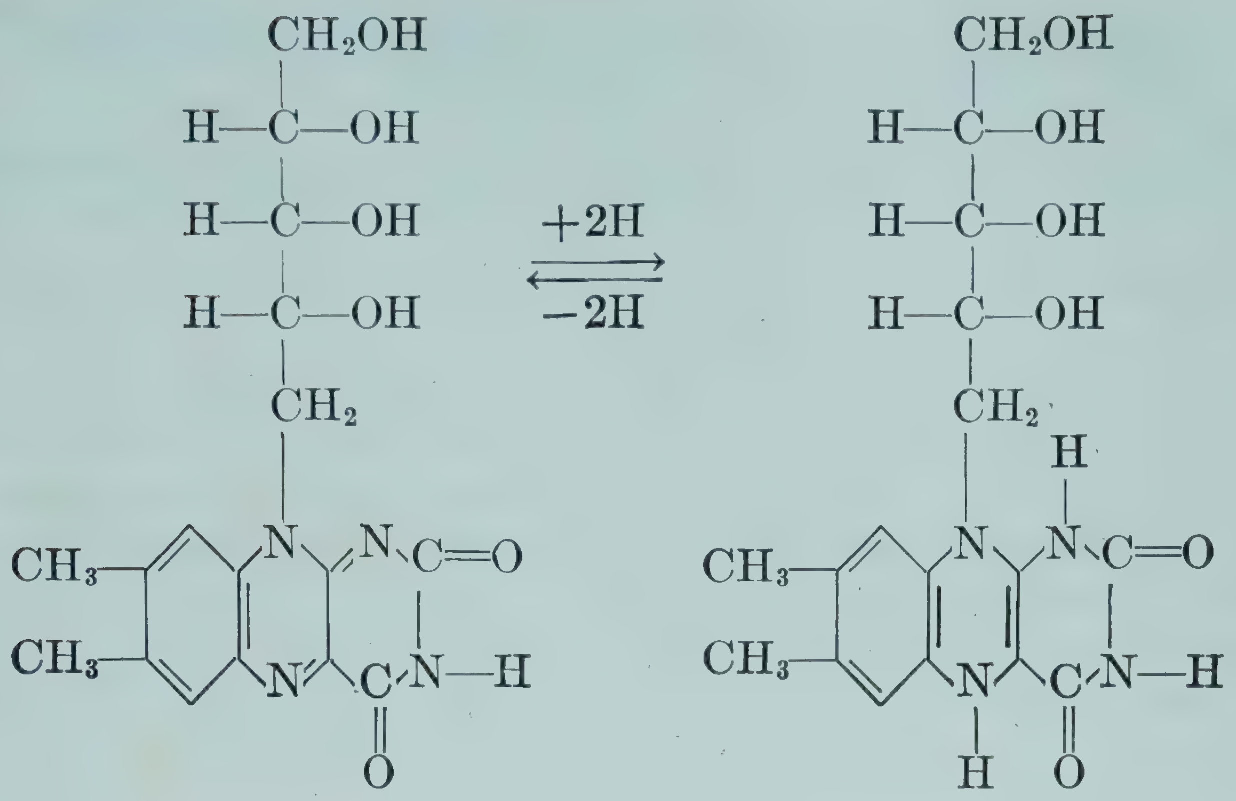
[120] See Appendix III.

[121] Bessey, Lowry, and Love: *J. Biol. Chem.*, **180**, 755 (1949).



Riboflavin **Leucoriboflavin**

Riboflavin may be converted to its mononucleotide, riboflavin phosphate, by treatment with phosphorus oxychloride, or by suspending it in a phosphate buffer with intestinal epithelial powder.

Determination of Riboflavin. Chemical determinations of riboflavin are based upon colorimetric[122] and fluorometric[123] procedures. In the analysis of food materials, colored and fluorescent compounds often interfere not only with the colorimetric determination of riboflavin but also with the fluorometric procedure in which the activating and fluorescent light are quenched. Some interfering pigments may be destroyed in the chemical test by oxidation with potassium permanganate or by reduction with stannous chloride. Fluorescence measurement before and after reduction of riboflavin by treatment with sodium hydrosulfite has been employed to correct for the fluorescence produced by interfering substances. Correction may be made for the quenching effect of foreign pigments or other factors by the internal standard procedure. One method for the chemical determination of riboflavin[124] involves the conversion of the vitamin by irradiation in alkaline solution to lumiflavin. The latter is extracted from acid solution by chloroform and then determined colorimetrically or fluorometrically.

The microbiological determination of riboflavin depending upon the growth stimulation of *Lactobacillus casei* is more generally applicable to the determination of the vitamin in foods than the chemical methods. The microbiological procedure is sensitive and specific, and has been adopted as an official method by the U.S. Pharmacopeia. It has been found that the growth of *Lactobacillus casei* in the presence of riboflavin is stimulated by suspended particles such as fat and starch. It is therefore essential to conduct the test on crystal-clear extracts. These are sometimes difficult to obtain when starchy samples are tested. Digestion with "Mylase" or "Taka-Diastase" is often helpful.

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[122] Warburg and Christian: *Biochem. Z.*, **257**, 492 (1933).

[123] Hodson and Norris: *J. Biol. Chem.*, **131**, 621 (1939); Scott, Hill, Norris, and Heuser: *J. Biol. Chem.*, **165**, 65 (1946).

[124] Kuhn, Wagner-Jauregg, and Kaltschmitt: *Ber.*, **67**, 1452 (1934).

Modified Microbiological Method of Snell and Strong:[125] ***Principle.*** Riboflavin is determined by measurement of the growth stimulation of *Lactobacillus casei*. The acid produced by the microorganism is determined by titration with sodium hydroxide.

Procedure: Preparation of Sample. **Conduct all operations in dim light. Weigh out a sample containing approximately 200 μg. riboflavin and heat in an autoclave with 400 ml. 0.1 N hydrochloric acid for 30 minutes at 15 pounds pressure. Cool and adjust to pH 4.5 with 1 N sodium hydroxide. Dilute to 1000 ml. and filter through a fine filter paper which does not adsorb riboflavin. Adjust a 100-ml. aliquot of the clear filtrate to pH 6.8 with 1 N sodium hydroxide solution and dilute to 200 ml. Filter again if the solution is not clear.**

Prepare the following solutions:

Yeast Extract Solution. **Heat a suspension of 500 g. fresh, starch-free baker's yeast in 5 liters of distilled water for 2 hours in flowing steam; then autoclave for 40 minutes at 15 pounds pressure. Allow the suspension to settle, filter, and evaporate the filtrate to 125 ml. under reduced pressure below 50° C.**

Yeast Supplement Solution. **Add 125 ml. of an aqueous solution containing 38 g. lead subacetate to 125 ml. of yeast extract solution. Filter and adjust the filtrate to pH 10 with ammonia. Filter and adjust the pH to 6.5 with glacial acetic acid. Treat the solution with hydrogen sulfide, filter off the lead sulfide, and dilute the filtrate to 250 ml. with water. Preserve under toluene in a refrigerator.**

Photolyzed Peptone Solution. **Dissolve 40 g. peptone in 250 ml. of distilled water, and 20 g. sodium hydroxide in 250 ml. distilled water. Mix the solutions and irradiate 6 to 10 hours in a crystallizing dish (diameter 24 cm.) 1 foot from a 100-watt bulb fitted with a reflector; then allow the mixture to stand for the remainder of a 24-hour period, keeping the temperature below 25° C. during the entire treatment. Adjust to pH 7 with glacial acetic acid, add 7 g. anhydrous sodium acetate, and dilute to 800 ml. Store under toluene in a refrigerator.**

Cystine Solution. **Dissolve 1 g. L-cystine in 20 ml. 10 per cent hydrochloric acid and dilute to 1000 ml. with distilled water. Store under toluene in a refrigerator not below 10° C.**

Salt Solution A. **Dissolve 25 g. monobasic potassium phosphate and 25 g. dibasic potassium phosphate in distilled water and dilute to 250 ml.**

Salt Solution B. **Dissolve 10 g. magnesium sulfate, 0.5 g. sodium chloride, 0.5 g. ferrous sulfate, and 0.5 g. manganese sulfate in distilled water, and dilute to 250 ml.**

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[125] Snell and Strong: *Ind. Eng. Chem., Anal. Ed.*, 11, 346 (1939). See also U.S. Pharmacopeia XIV.

Reference Standard Solution. **Dissolve 50 mg. U.S.P. riboflavin reference standard in 500 ml. distilled water containing 1 ml. glacial acetic acid. Preserve this stock solution under toluene in an amber bottle in the refrigerator. For each set of assays, dilute 1 ml. of this solution to 1000 ml. with distilled water. This solution contains 0.1 μg. riboflavin per ml.**

Basal Medium Stock Solution. **Dissolve 15 g. anhydrous dextrose in a mixture of 50 ml. photolyzed peptone solution, 50 ml. cystine solution, 5 ml. yeast supplement solution, 2.5 ml. salt solution A, and 2.5 ml. salt solution B. Adjust to pH 6.8 with 1 N sodium hydroxide and dilute to 250 ml.**

Preparation of the Stock Culture. **Dilute 10 ml. yeast extract solution to 100 ml. with distilled water, and add 1 g. anhydrous dextrose and 1.5 g. agar. Heat on a steam bath to dissolve the agar. Add approximately 10 ml. portions of the hot solution to test tubes, plug with nonabsorbent cotton and sterilize in an autoclave at 15 pounds pressure for 20 minutes. Allow to cool in an upright position. Prepare several stab cultures of *Lactobacillus casei* (American Type Culture Collection No. 7469) and incubate for 16 to 24 hours at any constant temperature between 30° and 37° C. Finally store in a refrigerator. Prepare a fresh stab every week, and do not use for inoculum if more than two weeks old.**

Preparation of the Inoculum. **To each of two tubes containing 5 ml. of the basal medium stock solution, add 5 ml. distilled water containing 1 μg. riboflavin. Autoclave at 15 pounds pressure for 20 minutes and cool. To one of the tubes make a transfer of cells from the stock culture of *Lactobacillus casei* and incubate for 16 to 24 hours at any constant temperature between 30° and 37°. Transfer 1 drop of this culture to the second tube and incubate again for 16 to 24 hours. Centrifuge the culture under aseptic conditions and decant the supernatant liquid. Suspend the cells in 10 ml. sterile isotonic solution of sodium chloride.**

Assay. **In duplicate tubes, 16 by 150 mm. in size, place respectively 0.5, 1.0, 1.5, and 2.0 ml. of the extract of the test material. To each add 5 ml. of the basal medium stock solution and sufficient distilled water to bring the volume in each tube to 10 ml. Prepare a similar set of duplicate tubes containing respectively 0.00, 0.05, 0.10, 0.15, 0.20, 0.30, and 0.50 μg. standard riboflavin. Mix the solutions thoroughly, plug the tubes with nonabsorbent cotton, and autoclave at 15 pounds pressure for 20 minutes. Cool aseptically, add 1 drop of inoculum to each tube, and incubate for 72 hours at any constant temperature between 30° and 37° C. Keep all the tubes in darkness or semidarkness during their preparation and incubation, and protect against contamination by foreign microorganisms. Transfer the contents of each tube to a small Erlenmeyer flask, using a fixed volume of distilled water for rinsing. Titrate with 0.1 N sodium hydroxide using bromothymol blue as the indicator.**

CALCULATION. On ordinary graph paper, plot the average titrations in ml. of 0.1 N sodium hydroxide against μg. of riboflavin in the series of standard tubes. From this standard curve, estimate the riboflavin content of each ml. of the test solution in each duplicate set of tubes. Calculate the riboflavin content of the test material from the average values obtained from not less than three sets of these tubes which do not vary by more than ±10 per cent from the average.

Comment. The microbiological method for the determination of riboflavin employing *Lactobacillus casei* is highly sensitive and shows a good degree of specificity for the vitamin. If desired, turbidity in place of acidimetric titration may be used to measure response. For measurement of turbidity follow the directions given in the Microbiological Assay for Vitamin B_{12}, p. 1210, beginning with the fifth paragraph under the heading *Procedure* and substituting riboflavin for cyanocobalamin. For this procedure it may be necessary to reduce the level of riboflavin used in the standard and sample tubes.

Fluorometric Method of Arnold:[126] ***Principle.*** The fluorometric procedure for the determination of riboflavin depends upon the extraction of the vitamin with dilute acid, filtration, treatment of the filtrate with permanganate and hydrogen peroxide to destroy interfering pigments, and measurement of the fluorescence. The vitamin content of the extract is evaluated by means of an internal standard.

Procedure. **Suspend a finely ground sample containing approximately 5 μg. riboflavin in 75 ml. 0.1 N sulfuric acid. Heat in an autoclave for 15 minutes at 15 pounds pressure, or in a boiling water bath for 45 minutes with intermittent shaking. Cool the suspension and adjust the pH to 4.3 with 2.5 M sodium acetate. Dilute to 100 ml., shake well, and filter through Whatman No. 1 or No. 40 filter paper. Discard the first 15 ml. filtrate. Treat 60 ml. of the filtrate with 2 ml. 4 per cent potassium permanganate solution. After 3 minutes, discharge the permanganate color with freshly prepared 3 per cent hydrogen peroxide solution. Break the froth with a few drops of acetone, and dilute to 65 ml. with distilled water. Mix and filter. Pipet out two 15-ml. portions of the filtrate. To one, add 1 ml. distilled water and measure the fluorescence (*A*) (see p. 1163). To the other, add 1 ml. riboflavin standard solution containing 1 μg. of the vitamin, and measure the fluorescence (*B*). Obtain the blank (*C*) after adding exactly 20 mg. of sodium hydrosulfite**[127] **to A or B.**

CALCULATION. Obtain the riboflavin content of the sample using the formula,

$$\frac{A - C}{B - A} \times \frac{1}{15} \times \frac{65}{60} \times \frac{100}{G} = \mu\text{g. riboflavin per g. of sample}$$

where G is the weight of the sample taken and A, B, and C are as indicated above.

Comment. The fluorometric method may be employed for the determination of the riboflavin content of yeast, white flour, bread, milk powder, and similar products. However, for samples which yield highly pigmented extracts, or for materials containing less than 1 μg. of riboflavin per g. of solids, the microbiological procedure is preferred.

Determination of Riboflavin in Urine (Flurorometric Method of Najjar):[128] ***Principle.*** The riboflavin is extracted with acetic acid pyridine butanol mixture after interfering urinary pigments are oxidized with permanganate. The concentration of riboflavin in the extract is measured fluorometrically.

Procedure. **Collect a 24-hour urine sample in a brown glass bottle containing 20 ml. of 10 per cent sulfuric acid. Since riboflavin is destroyed by light, conduct all of the following operations in semidarkness. Measure the volume.**

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[126] Arnold: *Cereal Chem.*, 22, 455 (1945).

[127] Sodium hydrosulfite (highest purity) should be stored so as to avoid undue exposure to light or air.

[128] Najjar: *J. Biol. Chem.*, 141, 355 (1941).

Transfer an aliquot containing approximately 5 μg riboflavin to a 100-ml. centrifuge cup, and add sufficient water to make the volume 10 ml. Add 2 ml. glacial acetic acid and 4 ml. colorless pyridine and mix. For each ml. of urine, add 2 drops 4 per cent $KMnO_4$. After exactly 1 minute, decolorize the excess permanganate with a few drops of freshly prepared 3 per cent hydrogen peroxide solution. Add 10 g. anhydrous sodium sulfate, then 20 ml. *n*-butanol. Shake vigorously for 5 minutes. Centrifuge.

Determine the fluorescence of the supernatant solution, that of a standard containing 5 μg. riboflavin, and of a water blank, the latter two likewise carried through the entire procedure.

For these measurements, a Pfaltz and Bauer fluorophotometer may be employed. The source of light should be a mercury vapor lamp equipped with a dark blue filter (Jena, No. BG12) and a yellow filter (Jena No. GG3). An orange filter (Jena, No. OG1) should be placed between the fluorescent solution and the photocell. Set the diaphragm so that the galvanometer reads 50 when the extract of the 5 μg. standard is in the instrument.

At the concentrations recommended in this procedure, the fluorescence of a riboflavin solution is directly proportional to its concentration. With most instruments, the galvanometer deflection is proportional to the fluorescence. Establish the linearity of the instrument employed by measuring the fluorescence of standard aqueous solutions of riboflavin as follows:

Dissolve 50.0 mg. riboflavin in 1000 ml. of hot water in the dark. Cool. Prepare aqueous dilutions containing 0.00, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, and 0.40 μg. riboflavin per ml. Set the instrument at 100 with the most concentrated standard and measure the fluorescence of the other solutions. Plot the galvanometer readings against concentration of the vitamin on ordinary graph paper. Check the setting of the instrument before each reading.

CALCULATION. If the relation is linear, calculate the riboflavin content of the 24-hour sample by means of the following formula:

$$\frac{U - C}{50 - C} \times 5 \times \frac{V}{A} = \mu\text{g. riboflavin per 24-hour sample}$$

where U is the galvanometer reading of the unknown, C is that of the reagent blank, V is the volume of the 24-hour sample in ml., and A that of the aliquot taken for test.

If the curve obtained above is not linear, standard solutions containing 0, 2, 4, 6, and 8 μg. riboflavin must be carried through the entire procedure and the concentration of riboflavin in the unknown determined from a standard curve. This value, multiplied by $\frac{V}{A}$, gives the amount of riboflavin in the 24-hour urine sample.

Comment. The fluorometric method for the determination of riboflavin in urine, as described above, measures in addition uroflavin, a fluorescent metabolite of the vitamin. For diagnostic purposes, however, the total fluorescence following the ingestion of a test dose of the vitamin is a good index of the nutritional state with respect to riboflavin.

BIOLOGICAL ASSAY OF RIBOFLAVIN

The essential feature in the biological assay for riboflavin is to select a basal ration which includes all of the required factors of the vitamin B complex with the sole exception of riboflavin. In the past the practice has been to supplement a basal ration otherwise free from all sources of the vitamin B complex with alcoholic extracts of wheat germ or of rice polishings (so-called tiki-tiki). However, such diets were undoubtedly deficient in more respects than riboflavin alone. Since crystalline preparations of each of the known B vitamins are now available, it is possible to supplement an otherwise adequate diet of purified components with all of the factors of the B complex except the one under assay.

There is at present no officially recognized procedure for the biological assay of riboflavin. Procedures employing both the rat and the chick have been described. The curative rat assay is more widely employed in the evaluation of foods or pharmaceutical products for human use. The customary procedure is to place rats on the riboflavin-free basal diet at weaning, making observations of weight changes at semiweekly intervals. Cessation of growth is one of the earliest symptoms of riboflavin depletion, and if the deprivation is complete, it is usually followed by decline in weight and death without the appearance of characteristic symptomatology. If the deficiency is prolonged (as may occur if the basal diet is not completely devoid of riboflavin) the symptoms of ariboflavinosis appear. These include bilaterally symmetrical patches of baldness around the nose, neck, back, and abdomen, and corneal vascularization, and if the deficiency is particularly prolonged, cataracts are observed. It is interesting to point out that riboflavin-deficient rats are particularly susceptible to pediculosis. The occurrence of alopecia is noted in deficiencies of other factors of the B complex—e.g., biotin. Whether this phenomenon has a common origin in deficiencies of various members of this group of vitamins is not known.

After three to four weeks on the riboflavin-free diet, the weight of the rat reaches a plateau or decline and at this point the material to be assayed is fed as a supplement to the basal ration. Comparable groups of animals receive graded dosages of crystalline riboflavin at levels which result in a graded dose-response curve. For example, doses of 1, 2, 4, 8, and 16 μg. of crystalline riboflavin may be fed daily. Together with a resumption of growth, restoration of the body hair occurs over the denuded areas. Potency may be estimated by comaprison of the log dose vs. growth response data for the test material with those for the standard (*cf.* calculation in vitamin A growth assay, Appendix VI).

NIACIN (NICOTINIC ACID)

The most common acute deficiency disease in this country is pellagra. Before the discovery of its relationship to niacin, this disease was responsible for thousands of deaths annually in the South. Pellagra occurs in endemic and epidemic form in many other countries including the Soviet Union, Egypt, Italy, Spain, and the Balkan countries. Epidemic pellagra follows any serious interruption of food supply or intake, especially after war, famine, disease, or economic depression.

Pellagra has been known for many centuries and was described as early as 1735 by Casal of Spain. That the disease was of dietary origin was not recognized until 1912 when Goldberger, Waring, and Willets[129] demonstrated that pellagra could be prevented by an adequate diet. At about the same time, nicotinic acid, a compound synthesized as early as 1867,[130] was isolated by Funk while attempting to purify the antiberiberi vitamin. He failed to recognize the relationship between nicotinic acid and pellagra but noted its beneficial effects when fed along with "vitamine B."[131] Deficiency syndromes analogous to human pellagra were demonstrated in dogs[132] in 1917 and in albino rats[133] in 1926. It was soon recognized that those foods which were effective for the cure of canine blacktongue were also effective for the cure of pellagra.[134] Following the discovery in 1937 of the importance of the amide of nicotinic acid (niacinamide) in the nutrition of certain unicellular organisms,[135] the therapeutic effects of nicotinic acid in the treatment of blacktongue[136] and of niacinamide in pellagra[137] were demonstrated.

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[129] Goldberger, Waring, and Willets: *U.S. Pub. Health Repts.*, **30**, 3117 (1915).

Physiological and Clinical Aspects of Niacin. In niacin deficiency, the demonstrable tissue lesions are preceded by functional disturbances which are characteristic of deficiencies of a number of the B vitamins. These include weakness, anorexia, indigestion, diarrhea, and mental and emotional disturbances. Though pellagra is generally complicated by deficiencies of other B vitamins, certain lesions of the skin, the digestive tract, and the nervous system (the "three D's"—dermatitis, diarrhea, dementia) have been attributed specifically to deficiency of niacin. The skin lesion is an erythema, generally bilaterally symmetrical, which affects the back of the hands, the knees, elbows, dorsum of the feet, and ankles. Exposure to the sun increases the severity of the lesions. In chronic pellagra the affected areas become permanently pigmented and either atrophied or roughened and thickened. Epithelial lesions are also noted in the digestive tract, particularly on the tongue (glossitis) and in the rectum. The organic nerve lesions of pellagra include myelin degeneration of the spinal column fibers and degeneration of the axis cylinders of pyramidal cells of the cortex. Mild mental disturbances are common in pellagra, and acute delirium and dementia occur in severe cases.

Though anemia is not generally recognized as a symptom of pellagra, it is frequently accompanied by a macrocytic anemia. Since this condition responds to pteroylglutamic (folic) acid, its occurrence in pellagra may be due to the multiple nature of the clinically observed condition. Dogs with blacktongue also show anemia which responds to the administration of niacin.[138]

Dramatic cures of pellagra occur upon the administration of niacinamide. Therapeutic doses of 50 to 500 mg. of niacinamide per day cause the disappearance of glossitis in 24 hours and of lesions of the tongue and lips in three to five days. Since uncomplicated niacin deficiency is comparatively rare, other members of the vitamin B complex are usually also administered in treating the disease.

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[130] Huber: *Liebig's Ann. Chem.*, **141**, 271 (1867).

[131] Funk: *J. Physiol.*, **46**, 173 (1913); *Brit. Med. J.*, **1**, 814 (1913). Drummond and Funk: *Biochem. J.*, **8**, 594 (1914).

[132] Chittenden and Underhill: *Am. J. Physiol.*, **44**, 13 (1917).

[133] Goldberger and Lillie: *U.S. Pub. Health Repts.*, **41**, 1025 (1926).

[134] Aykroyd and Roscoe: *Biochem. J.*, **23**, 483 (1929).

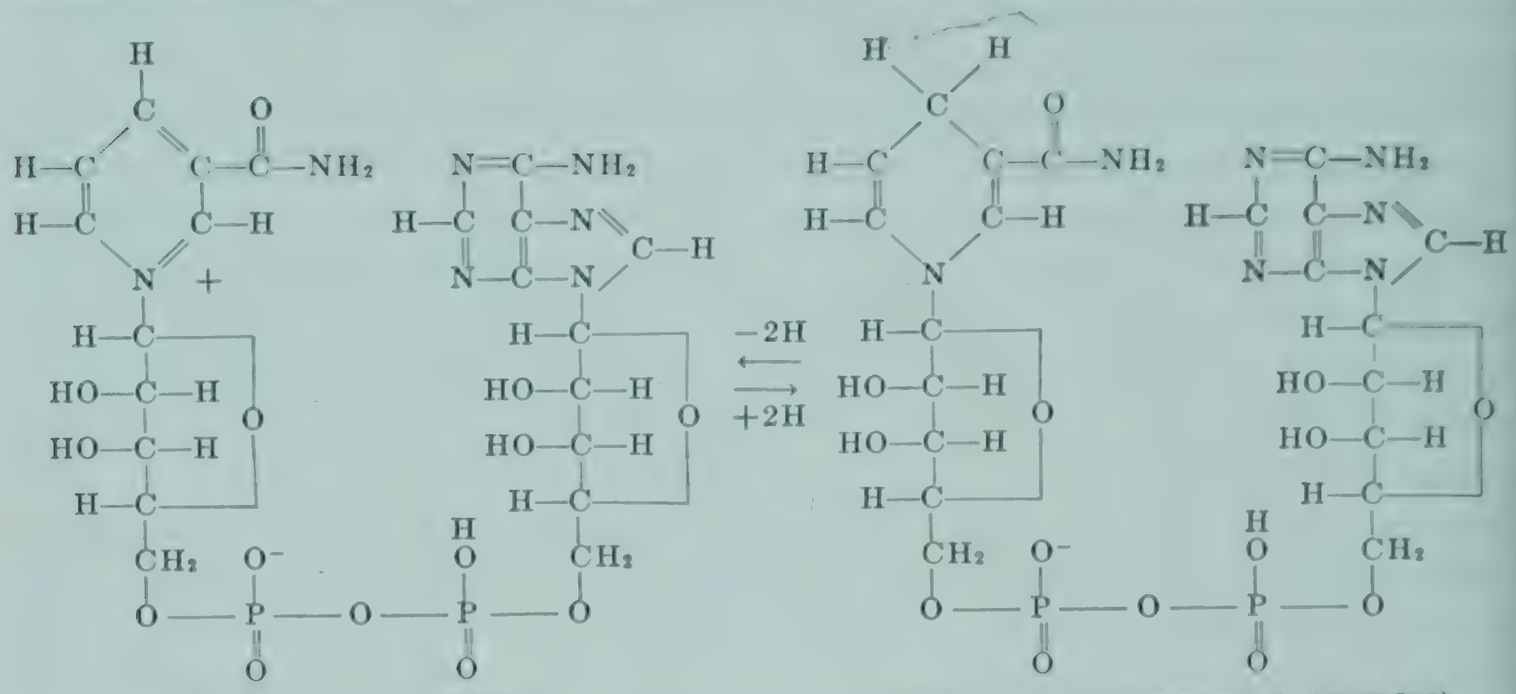
[135] Knight: *Biochem. J.*, **31**, 731 (1937).
Muller: *J. Biol. Chem.*, **120**, 219 (1937).

[136] Elvehjem, Madden, Strong, and Woolley: *J. Am. Chem. Soc.*, **59**, 1767 (1937); *J. Biol. Chem.*, **123**, 137 (1938).

[137] Fouts, Helmer, Lepkovsky, and Jukes: *Proc. Soc. Exp. Biol. Med.*, **37**, 405 (1937).

[138] Handler and Featherston: *J. Biol. Chem.*, **151**, 395 (1943).

Niacinamide is an essential constituent of diphosphopyridine and triphosphopyridine nucleotides (DPN and TPN, also known as coenzymes I and II, or codehydrogenases I and II), which participate in a large number of physiologically important oxidation reactions. These coenzymes occur in practically all cells and have been synthesized from niacin and niacinamide *in vitro* by nucleated cells. The structures of coenzyme I and of its reduction product, reduced coenzyme I, are shown below:



Coenzyme I (DPN) **Reduced coenzyme I (DPNH)**

The structure of reduced coenzyme I is based upon the evidence presented by Pullman *et al.*[138a] that reduction involves saturation of the carbon atom para to the nitrogen in the pyridine ring, rather than ortho to it, as had been previously thought. Note furthermore that reduction involves the addition of only one hydrogen atom to a molecule of coenzyme; the second hydrogen furnishes an electron to the quaternary N and becomes a hydrogen ion. Hence the stoichiometry of the reaction is as follows:

$$\underset{\textbf{Coenzyme I}}{\mathrm{DPN}^+} + 2\mathrm{H} \rightleftharpoons \underset{\textbf{Reduced Coenzyme I}}{\mathrm{DPNH}} + \mathrm{H}^+$$

TPN differs from DPN in that it contains an extra phosphoric acid group probably linked to the 2′ position of the ribose moiety of adenosine. These coenzymes, in the presence of specific proteins, catalyze physiological oxidation reactions. Among the 35 different known reactions in which DPN participates are the oxidation of alcohol to acetaldehyde, of glucose to gluconic acid, of malic acid to oxalacetic acid, of lactic acid to pyruvic acid, and of glycerophosphate to phosphoglyceraldehyde. Among the enzyme reactions catalyzed by TPN are the conversion of Robison's ester (glucose-6-monophosphate) to phosphohexonic acid and of glutamic acid to iminoglutamic acid. These enzymatic reactions are all reversible, and the reduced coenzymes may combine with their apoenzymes to catalyze physiological reductions. In practice there is a dynamic equilibrium which depends on the relative concentrations of the reduced and oxidized forms of the substrates and coenzymes and on other conditions. *In vitro* experiments indicate that reduced coenzymes I and II may be reoxidized by flavoprotein enzymes, and that coenzyme II may be converted to I by a phosphatase.[139] Both coenzymes are inactivated by enzymatic destruction when cells of brain, liver, kidney, or muscle are ruptured. In contrast to the riboflavin coenzymes, which are present in flavoproteins in simple numerical proportion to their apoenzymes, the niacinamide coenzymes are found in great excess and are bound only loosely to the protein moieties. The niacinamide prosthetic groups have been designated mobile coenzymes. DPN can be hydrolyzed by dilute alkali, the cleavage occurring between the niacinamide and ribose moieties. Similar splitting can be induced by enzymes (designated DPN nucleosidases) found in brain tissue[140] and in *Neurospora.*[141]

[138a] Pullman, San Pietro, and Colowick: *J. Biol.Chem.*, **206**, 129 (1954).

That dietary niacin is important in the maintenance of physiological enzyme systems is indicated by the positive correlation between the intake of the vitamin and the concentration of the coenzymes in the muscles.[142] Niacin is essential to the physiology of animals, plants, and microorganisms. All plants and some microorganisms synthesize the vitamin. Others—e.g., *C. diphtheriae,* lactic acid bacteria, and *B. dysenteriae*—require an external source, and still others—e.g., *B. influenzae*—require an external source of the coenzymes. Though the mold *Neurospora* does not ordinarily require niacin, a mutant strain produced by irradiation requires an external supply of the vitamin. Whereas niacin is essential to all animals, certain species, e.g., the rat and the horse, do not require dietary sources because their intestinal flora synthesize enough to meet metabolic needs. A dietary supply is required by the cotton rat, dog, pig, rabbit, chick, monkey, and man. However, even in these species, significant contributions are made by intestinal microorganisms. Urinary excretion studies[143] show that human subjects on a constant diet excrete lower concentrations of N^1-methylnicotinamide (the principal metabolite of niacin and niacinamide found in human urine) after dosage with sulfaguanidine and succinylsulfathiazole which destroy intestinal bacteria. Microorganisms have been isolated from the human cecum[144] which synthesize niacin *in vitro.*

The amount of niacin required by animals from external sources depends upon the nature of the diet, particularly with regard to protein and carbohydrate. Endemic pellagra occurs almost exclusively in localities where corn is employed as a staple cereal. Populations which consume large quantities of rice rarely show pellagra even though their dietary intake of niacin is no more than 5 mg. per day, whereas maize-eaters develop pellagra despite much higher intakes.[145] Rats, which ordinarily synthesize their own niacin, show a typical niacin deficiency syndrome when fed a corn diet.[146] This may be prevented by adding either tryptophan or more protein. On an otherwise adequate diet, young growing pigs show little sign of niacin deficiency, though the amount of the vitamin ingested is small. A decrease in the protein of the ration, however, produces symptoms of niacin deficiency which may be prevented by administration of more of the vitamin.[147]

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[139] Euler and Adler: *Z. physiol. Chem.,* **252,** 41 (1938).

[140] Gore, Ibbott, and McIlwain: *Biochem. J.,* **47,** 120 (1950).

[141] Kaplan, Colowick, and Nason: *J. Biol. Chem.,* **191,** 473 (1951).

[142] Anderson, Teply, and Elvehjem: *Arch. Biochem.,* **3,** 357 (1944).

[143] Ellinger, Coulson, and Benesch: *Nature,* **154,** 270 (1944); Ellinger, Benesch, and Kay: *Lancet,* **1,** 432 (1945).

[144] Benesch: *Lancet,* **1,** 718 (1945).

Since niacin deficiency in white rats on a corn diet is prevented by the administration of tryptophan, it appears that the lack of this amino acid, which is present in only low concentrations in corn, is responsible for the apparent increased requirement of many species for niacin. This effect may possibly be attributed to changes in the intestinal flora resulting in the loss of microorganisms which require tryptophan from which they are capable of synthesizing niacin. Tryptophan serves as a precursor for the biosynthesis of niacin not only by these microorganisms but in animal tissues as well. Hence pellagra must be viewed as a dual deficiency of both tryptophan and niacin. The possible existence of a pellagragenic antivitamin (see Chapter 36) in corn has received consideration.

The effect of corn on the growth of white rats depends upon the nature of the carbohydrate ingested. When the latter is sucrose, considerable inhibition of growth occurs. However, when sucrose is replaced by glucose and dextrin, or partially replaced by lactose, the inhibition of growth does not occur.[148] Further evidence that the increased requirement for niacin is associated with the nature of dietary carbohydrate and with a diminished intake of tryptophan is provided by observations on white rats subsisting on a diet containing wheat gluten and gelatin, and deficient only in niacin and tryptophan. With sucrose as the source of carbohydrate, very poor growth resulted. The administration of tryptophan or niacin, or the substitution of glucose and dextrin for sucrose, restored normal growth.

The principal metabolite excreted in the urine of man, rats, dogs, and cats following the ingestion of niacin or niacinamide is N^1-methylnicotinamide. Some nicotinamide but only small amounts of nicotinic acid are also excreted. However, rabbits and guinea pigs excrete principally nicotinic acid and practically no N^1-methylnicotinamide.[149] The latter compound, after alkaline hydrolysis, reacts like niacin with cyanogen bromide and aniline, and because of its physical and chemical similarity to trigonelline (the betaine of N-methylniacin) was at first believed to be that compound.[150] N^1-methylnicotinamide has the following structure:

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[145] Aykroyd and Swaminathan: *Indian J. Med. Research*, **27**, 667 (1940).

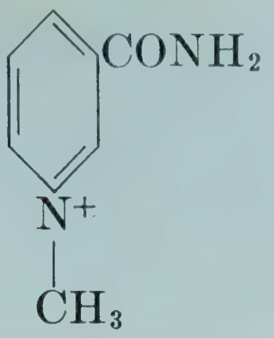
[146] Krehl, Teply, Sarma, and Elvehjem: *Science*, **101**, 489 (1945).

[147] Wintrobe, Stein, Follis, and Humphreys: *J. Nutrition*, **30**, 395 (1945).

[148] Krehl, Sarma, Teply, and Elvehjem: *J. Nutrition*, **31**, 85 (1946).

[149] Ellinger and Abdel Kader: *Biochem. J.*, **44**, 627 (1949).

[150] Melnick and Field: *J. Biol. Chem.*, **134**, 1 (1940); Melnick, Robinson, and Field: *Ibid.*, **136**, 145 (1940); Field, Melnick, Robinson, and Wilkinson: *J. Clin. Invest.*, **20**, 379 (1941).



N¹-Methylnicotinamide

Pellagrins were observed to excrete only small quantities of this metabolite. Early tests for the diagnosis of niacin deficiency were based upon the colorimetric determination of urinary "trigonelline" after the ingestion of a test dose of the vitamin. It was subsequently discovered[151] that administration of niacin or niacinamide was followed by excretion of a metabolite which was readily converted to a highly fluorescent substance by alkalinization and subsequent extraction with butanol. The urine of niacin-deficient subjects contained only small amounts of this compound, even after dosage with the vitamin. The metabolite was identified as N¹-methylnicotinamide[152] and found to be identical with the compound measured colorimetrically after alkaline hydrolysis which had been erroneously designated "trigonelline." Humans in a normal state of nutrition excrete approximately 20 per cent of their intake of niacinamide in the form of the N¹-methyl compound on a molar basis and approximately half of this amount when ingesting niacin.[153] The conversion of the latter to N¹-methylnicotinamide requires both an amidation and a methylation, whereas niacinamide requires only the latter step. *In vitro* studies[154] show that rat liver (but not kidney or muscle) is capable of converting niacinamide, but not niacin, to N¹-methylnicotinamide, the degree of conversion being increased by the addition of methionine.

The possibility that niacin or its amide acts physiologically in the form of N¹-methylnicotinamide has been investigated with conflicting results. Some observers have found that the compound is capable of curing blacktongue,[155] but this has been disputed by others.[156] In man, N¹-methylnicotinamide has been found to improve the dermatitis and glossitis of pellagra but simultaneously to aggravate the psychomotor symptoms.[157] The latter however, could be alleviated by the administration of thiamine and riboflavin. In view of these findings, the metabolic role of N¹-methylnicotinamide remains to be established.

Certain derivatives of niacin and niacinamide are biologically active in higher animals when administered orally. These include various esters, and the ureide and diethylamide of niacin. These compounds are probably hydrolyzed in the gastrointestinal tract to niacin and niacinamide. Trigonelline, the betaine of N-methylniacin, is biologically inactive. It occurs naturally in a number of foods, especially legumes, coffee, and tobacco. A synthetic analog of niacin, pyridine-3-sulfonic acid, has been found to inhibit the growth of microorganisms requiring niacin or its amide.[158] 3-Acetylpyridine does not inhibit microbial growth but induces niacin deficiency in mice, a species which normally does not require a dietary supply of niacin.[159] This compound, because of its chemical similarity to niacin, probably acts by competition with the vitamin in physiological enzyme systems (see Chapter 36).

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[151] Najjar and Wood: *Proc. Soc. Exptl. Biol. Med.*, **44**, 386 (1940); Najjar: *Bull. Johns Hopkins Hosp.*, **74**, 392 (1944).

[152] Huff and Perlzweig: *Science*, **97**, 538 (1943); *J. Biol. Chem.*, **150**, 395, 483 (1943).

[153] Hochberg, Melnick, and Oser: *J. Biol. Chem.*, **158**, 265 (1945).

[154] Perlzweig, Bernheim, and Bernheim: *J. Biol. Chem.*, **150**, 40 (1943); Ellinger: *Biochem. J.*, **42**, 175 (1948).

[155] Najjar, Hall, and Deal: *Bull. Johns Hopkins Hosp.*, **76**, 83 (1945).

[156] Tepley, Krehl, and Elvehjem: *Proc. Soc. Exptl. Biol. Med.*, **58**, 169 (1945).

[157] Vance: *Bull. Johns Hopkins Hosp.*, **77**, 393 (1945).

Wheat bran and other natural materials contain a compound which, upon heating with strong acid or alkali, behaves like niacin toward *Lactobacillus arabinosus*.[160] This compound is extracted from natural materials by heating with water or dilute acid, but must be subsequently hydrolyzed with strong acid or with alkali before it can stimulate the microorganism. Concentrates of the unhydrolyzed material have been prepared and found to be inactive when fed to chicks. After hydrolysis, the compound meets the niacin requirements of both the chick and the dog. Nikethamide ("Coramine"), niacin-diethylamide, exhibits similar behavior[161] in that it is inactive for *L. arabinosus* but is active after heating with alkali. Nikethamide is biologically active in higher animals.

Large doses of niacin dilate the superficial blood vessels. The administration of 50 mg. or more orally or of 10 mg. intravenously to humans not deficient in the vitamin produces flushing, itching, and burning of the skin, especially in areas subjected to pressure. Individuals vary greatly in their sensitivity to this reaction. The effect is observed only with the free acid and not with niacinamide, so that the latter is preferred for therapeutic purposes. The flushing reaction may be prevented by simultaneous administration of glycine which provides amino groups for conversion of the free vitamin to its amide.

Large doses of niacin or niacinamide are toxic to the rat because of the depletion of the methyl donor, methionine.[162] Niacinamide is more toxic than the free acid, since more N^1-methylnicotinamide is excreted when the amide is fed. The toxicity is seen only in animals which excrete N^1-methylnicotinamide after dosage with the vitamin. Large doses of niacinamide are not toxic to young rabbits and guinea pigs, which do not excrete the methylated derivative. The toxicity of large doses of the vitamin in rats may be prevented by feeding methionine, or choline plus homocystine (see p. 1030).

For further discussion of the clinical aspects of niacin, see the American Medical Association syllabus, p. 1291.

Storage and Synthesis of Niacin. No significant amount of niacin or its amide is stored passively in animal tissue. Higher concentrations are found in the liver, muscle, and kidney for the performance of metabolic functions. A decrease in the niacin intake of man is followed by a diminished concentration of the niacin coenzymes in the striated muscles, but has little effect on the coenzyme content of the erythrocytes.

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[158] McIlvain: *Brit. J. Exptl. Path.*, **21**, 136 (1940).

[159] Woolley: *J. Biol. Chem.*, **157**, 455 (1945).

[160] Andrews, Boyd, and Gortner: *Ind. Eng. Chem., Anal. Ed.*, **14**, 663 (1942); Krehl and Strong: *J. Biol. Chem.*, **156**, 1 (1944).

[161] Teply and Elvehjem: *Proc. Soc. Exptl. Biol. Med.*, **55**, 72 (1944).

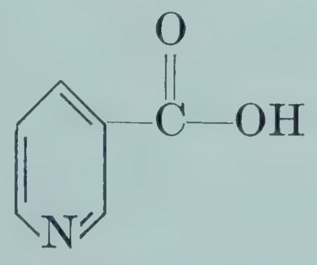
[162] Handler: *J. Biol. Chem.*, **154**, 203 (1944).

Biosynthesis of niacin by plants and microorganisms, the importance of the contribution by intestinal flora in the nutrition of animals, and the effect of other dietary factors have been discussed above.

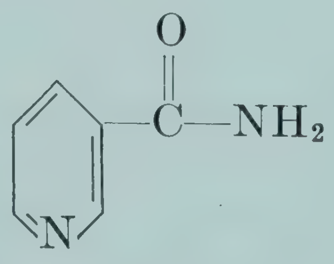
A number of practical syntheses for niacin and niacinamide are available for commercial use. The simplest of these include the nitric acid oxidation of nicotine obtained from tobacco or the oxidation of β-picoline or quinoline with potassium permanganate or in the vapor phase with air and metallic oxide catalysts. Niacinamide may be prepared from the free acid by heating with ammonia, from esters by treatment with alcoholic ammonia, or from 3-cyanopyridine by acid or alkaline hydrolysis in the presence of hydrogen peroxide and subsequent treatment with a quaternary ammonium resin.[163]

Distribution of Niacin.[164] In natural materials, niacin occurs predominantly as the amide, the form in which it is active biologically in enzyme systems. Good sources of the vitamin are liver, adrenal glands, fish, meats, whole wheat or rye, and enriched flour. The vitamin occurs in relatively high concentration as coenzyme I in yeast, red blood cells, and heart muscle. Fruits are poor sources of the vitamin, nuts are somewhat richer, and vegetables are variable, potatoes and legumes being generally higher than leafy varieties. Cow's milk may contain from about 1 mg. to as much as 5 mg. per quart, whereas human milk contains relatively less. Tea, coffee, and beer are good sources of niacin.

Chemistry of Niacin. Niacin (pyridine-3-carboxylic acid) is a white crystalline solid which melts at 236° C. and may be sublimed without destruction. The vitamin is stable to boiling in neutral, acid, or alkaline solution. The vitamin has an absorption maximum in the ultraviolet region at 385 mμ.



Niacin (Nicotinic Acid)



Niacinamide

Niacinamide crystallizes in light needles from benzene and melts at 129° C. It may be distilled at 150 to 160° at 5×10^{-4} mm. Niacinamide is more soluble in organic solvents than the free acid and, unlike the latter, may be extracted from water with ether. The amide has a characteristic absorption curve in the ultraviolet region with maxima at 210, 220, and 260 mμ. Niacinamide is readily hydrolyzed to free niacin by heating with acid or alkali.

Niacin and niacinamide react with cyanogen bromide in the presence of a primary or secondary amine to produce a compound having a yellow-green color. Colors are also formed with trigonelline, nicotinuric acid, and nicotine, as well as certain other pyridine compounds. This reaction is employed in the chemical determination of niacin. Since the amide produces only half the color of the free acid, it must first be hydrolyzed for the colorimetric test.

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[163] Galat: *J. Am. Chem. Soc.*, **70**, 3945 (1948).

[164] See Appendix III.

Coenzymes I and II (DPN and TPN) are colorless, water-soluble compounds, insoluble in organic solvents. Coenzyme II, however, is soluble in methanol–hydrochloric acid mixtures. Both coenzymes are relatively stable in acid solution but are readily decomposed by alkali. Coenzyme I has an absorption maximum at 260 mμ. It is nonfluorescent, optically active, $[\alpha]$ 546 mμ $= -70°$, and is not precipitated by lead acetate, but does produce an insoluble cuprous salt. Reduced coenzyme I has an absorption maximum at 340 mμ and fluoresces upon ultraviolet irradiation. This compound is stable in alkali but is destroyed by acid in which it reverts to the oxidized form which is non-fluorescent. Coenzyme II has an absorption maximum at 360 mμ. Its optical rotation $[\alpha]$ 546 mμ $= -29.4°$. Its cuprous salt is soluble but the coenzyme is precipitated by lead acetate.

Determination of Niacin. Accurate chemical and microbiological methods for the determination of niacin in natural materials are available. The chemical procedures involve reaction of the vitamin with cyanogen bromide in the presence of an amine. Extracts are prepared for chemical or microbiological assay by acid or alkaline hydrolysis, both to facilitate extraction and to hydrolyze niacinamide and a niacin precursor found in natural materials. In the chemical procedure, hydrolysis with acid results in the formation of furfural from the degradation of pentoses, which interferes with colorimetric reaction. This compound is separated from the niacin by adsorption of the vitamin on a hydrated aluminum silicate which does not adsorb the furfural. The niacin is subsequently eluted from the adsorbate by washing with alkali.

Because niacin and niacinamide have somewhat different physiological effects, it is sometimes desirable to determine their relative concentrations in mixtures. This may be accomplished chemically by time-reaction measurements conducted on the hydrolyzed and unhydrolyzed extracts.[165] The assay is based on the differences in the rate of formation of the yellow-green pigment with cyanogen bromide and aniline.

The microbiological determination of niacin is based upon the growth stimulation of *Lactobacillus arabinosus*. This organism responds equally to niacin and its amide. These compounds, however, may be differentiated microbiologically by destruction of the amide with bromine and potassium hydroxide (Hoffman degradation), converting it to β-amino-pyridine, which is microbiologically inert.[166] Differential microbiological assays may also be conducted to distinguish between the several metabolites of the vitamin, including niacin, its amide, nicotinuric acid, and N^1-methylnicotinamide. Two microorganisms are employed, *Lactobacillus arabino-*

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[165] Lamb: *Ind. Eng. Chem., Anal. Ed.*, **15**, 352 (1943); Melnick and Oser: *ibid.*, **15**, 355 (1943).

[166] Atkin, Schultz, Williams, and Frey: *J. Am. Chem. Soc.*, **65**, 992 (1943).

sus, which utilizes niacin, niacinamide, and nicotinuric acid, and *Leuconostoc mesenteroides*, for which only niacin is active.[167] Hydrolysis with 0.6 N sulfuric acid at 15 pounds pressure for one hour is also employed to convert the amide completely to free niacin without affecting nicotinuric acid. The microbiological assays are conducted in conjunction with the fluorometric test which measures only N^1-methylnicotinamide. Differential assays such as those described above show that the major part (about 90 per cent) of the niacin metabolites excreted after the ingestion of the vitamin appears as N^1-methylnicotinamide. Small amounts of niacin and niacinamide also occur in urine and somewhat larger amounts of the latter after the administration of large doses of the free acid. Human urine contains little if any nicotinuric acid except after large doses of free nicotinic acid.

The biochemical diagnosis of niacin deficiency is based upon the measurement of urinary excretion of N^1-methylnicotinamide.[168] A satisfactory clinical test involves measurement of the 6-hour urinary excretion of N^1-methylnicotinamide following the oral administration of 300 mg. niacinamide.[169]

Earlier clinical tests for the diagnosis of pellagra, based upon measurement of urinary "porphyrins," are not reliable since the color reaction has been found to be due to urorosein rather than to porphyrin and depends on the presence of other compounds in the urine.

Modified Colorimetric Method of the Research Corporation:[170] ***Principle.*** The method involves strong acid hydrolysis to convert derivatives to nicotinic acid, adsorption of the vitamin on Lloyd's reagent, clarification of the eluate with lead hydroxide, and reaction of nicotinic acid with cyanogen bromide and aniline to produce a yellow pigment which is measured photometrically.

Procedure: Preparation of the Sample. **Hydrolyze a sample of tissue or food containing 100–500 μg. of niacin by heating in a boiling water bath with 75 ml. 4 N hydrochloric acid for 30–40 minutes. Cool and dilute to 100 ml. Transfer 25 ml. of the suspension to a narrow (18-mm. diameter) centrifuge tube calibrated at the 26.5-ml. mark. Adjust the pH to 0.5–1.0 with 18 N sodium hydroxide using 0.1 per cent methyl violet as an outside indicator.[171] Cool, add 2.5 g. Lloyd's reagent,[172] and shake 1 minute. Centrifuge and discard the supernatant liquid. Wash the residue twice with 10 ml. 0.2 N sulfuric acid, each time centrifuging and discarding the supernatant. Add 0.5 N sodium hydroxide to the 26.5-ml. mark and break up the precipitate with a stirring rod. Immerse the tube in a boiling water bath for 5 minutes, stirring the contents occasionally. Cool and centrifuge. Transfer the supernatant to another tube, add 1.6 g. finely divided lead nitrate, and shake vigorously for 1 minute. If the solution is still alkaline, add more lead nitrate until acid.**

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[167] Johnson: *J. Biol. Chem.*, **159**, 277 (1945).

[168] Huff and Perlzweig: *J. Biol. Chem.*, **150**, 395 (1943).

[169] Goldsmith: *Arch. Internal Med.*, **73**, 410 (1944).

[170] Melnick: *Cereal Chem.*, **19**, 553 (1942).

[171] Use one drop of sample to one drop of indicator on a spot plate. Match with the blue color given by one drop of 0.2 N sulfuric acid.

[172] A hydrated aluminum silicate obtained from Eli Lilly and Co., Indianapolis, Ind. When suspended in water, 2.5 g. occupies a volume of 1.5 ml.

Centrifuge and pour the clear supernatant into another tube. Neutralize with tertiary potassium phosphate and 20 per cent phosphoric acid.

Colorimetric measurements are made in a photoelectric photometer. Two center settings are necessary, one for evaluating the residual color in the test solution, the other for the color developed in the chemical reaction. Set the photometer at 100 per cent transmittance with 3 ml. water and 7 ml. alcoholic buffer.[173] With this setting read a solution of 3 ml. of the test extract plus 7 ml. of alcoholic buffer (*A*). Now set the photometer at 100 per cent transmittance with the reagent blank: 3 ml. water, plus 6 ml. cyanogen bromide solution[174] plus 1 ml. aniline solution.[175] Remove the tube and record the center setting. To 3 ml. of the test extract, add 6 ml. cyanogen bromide, and 10 minutes later 1 ml. aniline; read the solution 5 minutes later using the center setting obtained with reagent blank (*B*). Similarly read a solution containing 3 ml. of test extract, 10 µg. of nicotinic acid in 0.1 ml. of an alcoholic solution, 6 ml. of cyanogen bromide, and 1 ml. of aniline (*C*).

CALCULATION. Convert galvanometer readings, *G*, to photometric density, *P.D.*, as follows:

$$P.D. = 2 - \log G$$

Then:

$$\frac{B - A}{1.01C - B} \times 10 \times \text{dilution factor} = \mu\text{g. nicotinic acid per g. sample}$$

Comment. Values obtained by the colorimetric method for nicotinic acid agree well with those obtained by microbiological assay of acid or alkaline extracts. Exhaustive aqueous extraction, without preliminary hydrolysis, removes all the nicotinic acid from cereal products but yields lower microbiological and chemical values. A nicotinic acid derivative other than nicotinamide is broken down by the hydrolytic procedure (see p. 1172).

Determination of N^1-Methylnicotinamide in Urine (Fluorometric Method of Hochberg, Melnick, and Oser):[176] ***Principle.*** The method involves adsorption of a small urine aliquot at pH 4.5 on a column of synthetic zeolite, elution with potassium chloride solution, alkalinization, extraction of the resulting compound with *n*-butanol, and measurement of the fluorescence.

Procedure: Preparation of Sample. **Collect a 24-hour urine sample in an amber bottle containing 20 ml. 10 per cent sulfuric acid. Measure the volume. Dilute a 6-minute aliquot[177] with acetate buffer[178] to 50 ml., and pass the solution through the special adsorption column shown in Fig. 278, following the directions given for the colorimetric determination of thiamine. Elute the N^1-methylnicotinamide with 15 ml. of a neutral 25 per cent solution of potassium chloride, passed down the wall of the hot condenser. Regenerate the**

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[173] Mix 980 ml. water, 15 ml. 15 per cent sodium hydroxide, 4 ml. 85 per cent phosphoric acid, and 167 ml. absolute ethyl alcohol.

[174] In a well-ventilated hood dissolve 20 g. cyanogen bromide crystals in 500 ml. cold water. Do not allow the crystals to come in contact with the skin since they produce serious burns.

[175] Four per cent redistilled aniline in absolute alcohol.

[176] Hochberg, Melnick, and Oser: *J. Biol. Chem.*, **158**, 265 (1945).

[177] Larger aliquots should not be taken, since incomplete absorption on the zeolite may result.

[178] Dissolve 7.5 g. sodium acetate in 1000 ml. of distilled water containing 1.0 ml. concentrated sulfuric acid. The pH is 4.5.

column by washing with water (see p. 1145). Pass a standard solution containing 50 μg. of N¹-methylnicotinamide chloride[179] in 50 ml. acetate buffer through the column in the same manner as the diluted urine samples.

Fluorometric Measurement. **Mix the eluate and pipet a 5-ml. aliquot into each of two 30-ml. separatory funnels. To one, the blank, add 1 ml. of water followed by 16.5 ml. *n*-butanol. To the other, the test, add 16.5 ml. butanol, then 1 ml. 15 per cent sodium hydroxide. Stopper the vessels immediately and shake vigorously for 3 minutes. Centrifuge for 0.5 minute and discard the aqueous layer. Clarify the butanol by shaking with approximately 1 g. anhydrous sodium sulfate. Allow the butanol extracts to stand in the dark for 15 minutes during which the fluorescence reaches a maximum, then read in a fluorometer with appropriate light filters. Use a solution of quinine sulfate containing 0.2 μg. per ml. in 0.1 N sulfuric acid to check the setting of the instrument.**

CALCULATION. Calculate the 24-hour urinary excretion of N¹-methylnicotinamide chloride, expressed in terms of niacinamide, using the formula,

$$50 \times \frac{G_u}{G_s} \times \frac{60}{M} \times 0.707 = \mu\text{g. niacinamide per 24 hr.}$$

G_u and G_s are the galvanometer deflections of the unknown and the standard, both corrected for their respective blanks. M is the period represented by the urinary aliquot, expressed in minutes, and 0.707 is the factor for conversion of N¹-methylnicotinamide chloride to niacinamide.

Comment. N¹-Methylnicotinamide is the principal derivative of niacinamide found in human urine. Average basal excretion values approximate 25–30 per cent of the dietary intake, with wide individual variations. The metabolite is excreted rapidly following ingestion of the vitamin, approximately 20 per cent appearing in the urine within 24 hours following dosage with 50–200 mg. niacinamide.

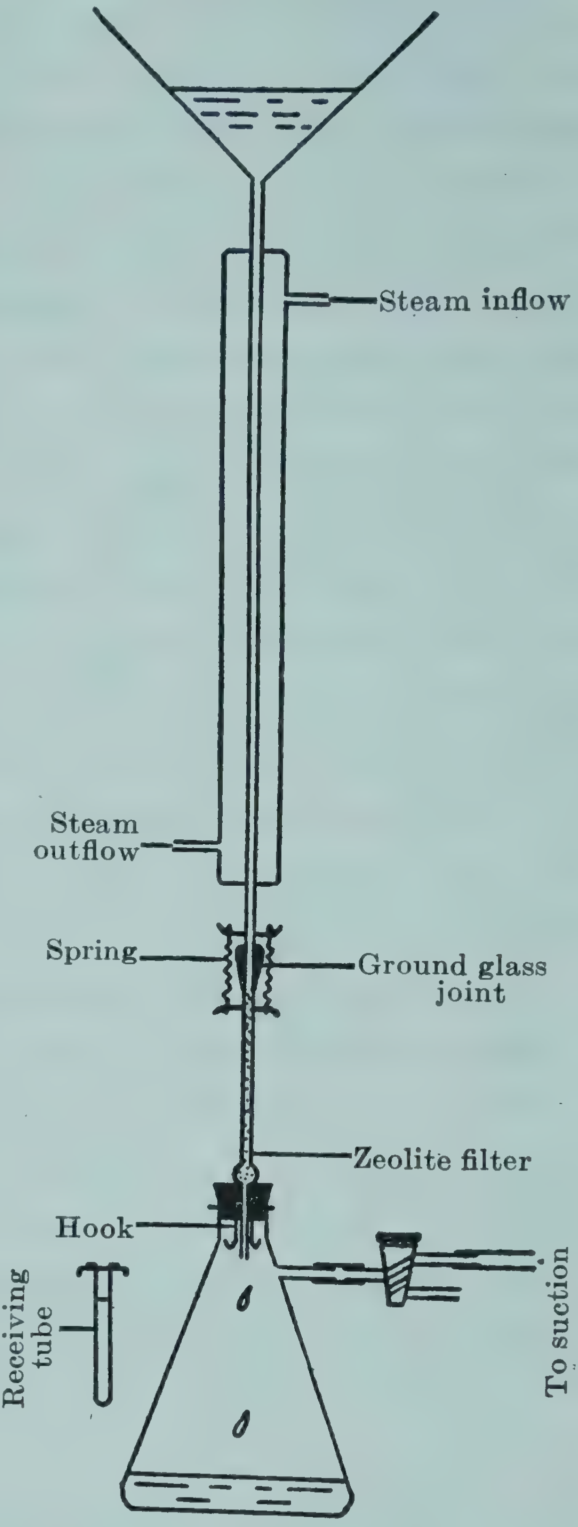

FIG. 278. Apparatus for the adsorption and elution of N¹-methylnicotinamide in urine.

ASSAY FOR NIACIN OR NIACINAMIDE

Microbiological Method of the U.S. Pharmacopeia XIV.[180]

TEST SOLUTION OF THE MATERIAL TO BE ASSAYED. Place the prescribed amount of the material to be assayed in a flask of suitable size, and proceed by one of the methods given below.[181]

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[179] The compound may be prepared by the method of Karrer, Schwazenbach, Benz, and Solmssen: *Helv. Chim. Acta*, **19**, 826 (1936); or it may be purchased from W. A. Taylor and Company, 7300 York Road, Baltimore, Md.

[180] Grateful acknowledgement for permission to reproduce this method is made to Dr. Lloyd C. Miller and the Board of Trustees of the U.S. Pharmacopeial Convention, Inc.

(*a*) *For dry or semi-dry materials that contain no appreciable amount of basic substances.* Add a volume of 1 N sulfuric acid equal in ml. to not less than ten times the dry weight of the material in g., but the resulting solution shall contain not more than 5.0 mg. of nicotinic acid per ml. If the material is not readily soluble, comminute it so that it may be evenly dispersed in the liquid, then agitate vigorously and wash down the sides of the flask with 1 N sulfuric acid.

Heat the mixture in an autoclave at 121° to 123° for 30 minutes and cool. If lumping occurs, agitate the mixture until the particles are evenly dispersed. Adjust the mixture to a pH of 6.8 with sodium hydroxide solution, dilute with water to make a final measured volume that contains approximately 0.1 μg. of nicotinic acid in each ml., and filter if the solution is not clear.

(*b*) *For dry or semi-dry materials that contain appreciable amounts of basic substances.* Add sufficient sulfuric acid solution to bring the pH of the mixture to between 5.0 and 6.0. Add such an amount of water that the total volume of liquid shall be equal in ml. to not less than ten times the dry weight of the sample in g., but the resulting solution shall contain not more than 5.0 mg. of nicotinic acid in each ml. Then add the equivalent of 10 ml. of 10 N sulfuric acid for each 100 ml. of liquid and proceed as directed under (*a*), beginning with the second sentence.

(*c*) *For liquid materials.* Adjust the material to a pH of 5.0 to 6.0 with either sulfuric acid solution or sodium hydroxide solution, and proceed as directed under (*b*), beginning with the second sentence.

STANDARD NICOTINIC ACID STOCK SOLUTION I. Dissolve 50 mg. of U.S.P. Nicotinic Acid Reference Standard, previously dried and stored in the dark in a desiccator over phosphorus pentoxide, in sufficient alcohol to make 500 ml. Store in a refrigerator. Each 1.0 ml. represents 100 μg. of U.S.P. Nicotinic Acid Reference Standard.

STANDARD NICOTINIC ACID STOCK SOLUTION II. To 100 ml. of stock solution I, add sufficient water to make 1000 ml. Store under toluene in a refrigerator. Each 1.0 ml. represents 10 μg. of U.S.P. Nicotinic Acid Reference Standard.

STANDARD NICOTINIC ACID SOLUTION. Dilute 10 ml. of stock solution II with sufficient water to make 1000 ml. Each 1.0 ml. represents 0.1 μg. of U.S.P. Nicotinic Acid Reference Standard. Prepare fresh standard solution for each assay.

BASAL MEDIUM STOCK SOLUTION

Acid-hydrolyzed Casein Solution	25 ml.
Cystine-Tryptophan Solution	25 ml.
Dextrose, Anhydrous	10 g.
Sodium Acetate, Anhydrous	5 g.
Adenine-Guanine-Uracil Solution	5 ml.
Riboflavin-Thiamine-Biotin Solution	5 ml.
p-Aminobenzoic Acid-Calcium Pantothenate-Pyridoxine Solution	5 ml.
Salt Solution A	5 ml.
Salt Solution B	5 ml.

Dissolve the anhydrous dextrose and sodium acetate in the solutions previously mixed, and adjust to a pH of 6.8 with sodium hydroxide T.S. [Test Solution]. Finally add water to make 250 ml.

ACID-HYDROLYZED CASEIN SOLUTION. Mix 100 g. of vitamin-free casein with 500 ml. of constant-boiling hydrochloric acid (approximately 20 per cent HCl), and reflux the mixture for 24 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution to a pH of 3.5 (±0.1) with sodium hydroxide T.S., and add water to make 1000 ml. Add 20 g. of activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal if the filtrate does not appear straw colored to colorless. Store under toluene in a refrigerator. Filter the solution if a precipitate forms upon storage.

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[181] The concentrations of the sulfuric acid and sodium hydroxide solutions used are not stated in each instance because these concentrations may be varied depending upon the amount of material taken for assay, volume of test solution, and buffering effect of material.

CYSTINE-TRYPTOPHAN SOLUTION. Suspend 4.0 g. of L-cystine and 1.0 g. of L-tryptophan (or 2.0 g. of D,L-tryptophan) in 700 to 800 ml. of water, heat to 70° to 80° and add 20 per cent hydrochloric acid, dropwise, with stirring, until the solids are dissolved. Cool, and add water to make 1000 ml. Store under toluene in a refrigerator at a temperature not below 10°.

ADENINE-GUANINE-URACIL SOLUTION. Dissolve 0.1 g. each of adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 5.0 ml. of 20 per cent hydrochloric acid, cool, and add water to make 100 ml. Store under toluene in a refrigerator.

RIBOFLAVIN-THIAMINE HYDROCHLORIDE-BIOTIN SOLUTION. Prepare a solution containing, in each ml., 20 μg. of riboflavin, 10 μg. of thiamine hydrochloride, and 0.04 μg. of biotin, by dissolving crystalline riboflavin, crystalline thiamine hydrochloride, and crystalline biotin (free acid) in 0.02 N acetic acid. Store, protected from light, under toluene in a refrigerator.

p-AMINOBENZOIC ACID–CALCIUM PANTOTHENATE–PYRIDOXINE HYDROCHLORIDE SOLUTION. Prepare a solution in neutral 25 per cent alcohol to contain 10 μg. of *p*-aminobenzoic acid, 20 μg. of calcium pantothenate, and 40 μg. of pyridoxine hydrochloride in each ml. Store in a refrigerator.

SALT SOLUTION A. Dissolve 25 g. of monobasic potassium phosphate and 25 g. of dibasic potassium phosphate in water to make 500 ml. Add 5 drops of hydrochloric acid and store under toluene.

SALT SOLUTION B. Dissolve 10 g. of magnesium sulfate, 0.5 g. of sodium chloride, 0.5 g. of ferrous sulfate, and 0.5 g. of manganese sulfate in water to make 500 ml. Add 5 drops of hydrochloric acid and store under toluene.

STOCK CULTURE OF LACTOBACILLUS ARABINOSUS 17–5. Dissolve 2.0 g. of water-soluble yeast extract in 100 ml. of water, add 0.5 g. of anhydrous dextrose, 0.5 g. of anhydrous sodium acetate, and 1.5 g. of agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Add approximately 10-ml. portions of the hot solution to test tubes, plug the tubes with cotton, sterilize in an autoclave at 121° to 123°, and allow the tubes to cool in an upright position. Prepare stab cultures in 3 or more of the tubes, using a pure culture of *Lactobacillus arabinosus* 17–5,[182] incubating for 16 to 24 hours at any selected temperature between 30° and 37° but held constant to within ±0.5°, and finally store in a refrigerator. Prepare a fresh stab of the stock culture every week and do not use for inoculum if the culture is more than 1 week old.

CULTURE MEDIUM. To each of a series of test tubes containing 5.0 ml. of the basal medium stock solution, add 5.0 ml. of water containing 1.0 μg. of nicotinic acid. Plug the tubes with cotton, sterilize in an autoclave at 121° to 123°, and cool.

INOCULUM. Make a transfer of cells from the stock culture of *Lactobacillus arabinosus* 17–5 to a sterile tube containing 10 ml. of culture medium. Incubate this culture for 16 to 24 hours at any selected temperature between 30° and 37° but held constant to within ±0.5°. The cell suspension so obtained is the inoculum.

Assay Procedure. **Prepare standard nicotinic acid tubes as follows: To duplicate test tubes, add 0.0 ml., 0.5 ml., 1.0 ml., 1.5 ml., 2.0 ml., 2.5 ml., 3.0 ml., 3.5 ml., 4.0 ml., 4.5 ml., and 5.0 ml., respectively, of the standard**

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[182] Pure cultures of *Lactobacillus arabinosus* 17–5 may be obtained from the American Type Culture Collection, 2029 M Street, N. W., Washington 6, D. C., as number 8014.

nicotinic acid solution. To each tube add 5.0 ml. of basal medium stock solution and water to make 10 ml.

Prepare tubes containing the material to be assayed as follows: To duplicate test tubes add, respectively, 1.0 ml., 2.0 ml., 3.0 ml., and 4.0 ml. of the test solution of the material to be assayed. To each tube add 5.0 ml. of basal medium stock solution and water to make 10 ml. After mixing, plug the tubes with cotton or cover with caps, and sterilize in an autoclave at 121° to 123°.[183] Cool, aseptically inoculate each tube with 1 drop of inoculum, and incubate for 72 hours at any selected temperature between 30° and 37°, but held constant to within ±0.5°. Contamination of the assay tubes with any foreign organism invalidates the assay.

Titrate the contents of each tube with 0.1 N sodium hydroxide, using bromothymol blue T.S. as the indicator, or to a pH of 6.8 measured electrometrically.

CALCULATION. Prepare a standard curve of the nicotinic acid standard titrations by plotting the average of the titration values expressed in ml. of 0.1 N sodium hydroxide for each level of the nicotinic acid standard solution used, against μg. of nicotinic acid contained in the respective tubes. From this standard curve, determine by interpolation the nicotinic acid content of the test solution in each tube. Discard any values of more than 0.40 or less than 0.05 μg. of nicotinic acid in each tube. Calculate the nicotinic acid content in each ml. of the test solution for each of the tubes. The nicotinic acid content of the test material is calculated from the average of the values obtained from not less than 6 of these tubes that do not vary by more than ±10 per cent from the average. If the titration values of less than 6 of these tubes containing the test solution are within the range of the titration values of the nicotinic acid standard tubes containing 0.05 to 0.40 μg. of nicotinic acid, the data are insufficient to permit calculation of nicotinic acid content of the test material. Titration values exceeding 1.0 ml. for the tubes of the standard nicotinic acid solution series containing 0.0 ml. of the solution indicate the presence of an excessive amount of nicotinic acid in the basal medium stock solution and invalidate the assay.

PYRIDOXINE AND RELATED COMPOUNDS (VITAMIN B_6)

Goldberger and Lillie in 1926 reported the development of a characteristic dermatitis, called acrodynia, in rats fed a diet deficient in what they regarded as the pellagra-preventive (P-P) factor. In 1934, recognizing the multiple nature of the vitamin B complex, P. György[184] designated the missing factor vitamin B_6, the rat pellagra-preventive factor, the deficiency being characterized by edema and denuding of the paws and the areas around the nose and mouth, and thickening of the ears. Lack of this vitamin was also shown to induce microcytic hypochromic anemia and neurologic lesions in dogs and pigs. Vitamin B_6 attracted so much attention that within one year, 1938, it was isolated independently by five groups—Lepkovsky; Keresztesy and Stevens; P. György; Kuhn and Wendt; and Itiba and Miti. In the following year, the chemical structure was elucidated and the compound was synthesized independently by Keresztesy, Stevens, Harris, Stiller, and Folkers in the United States and by Kuhn, Westphal, Wendt, and Westphal in Germany. The vitamin was named pyridoxine by the American investigators and adermin by the Germans.

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[183] Overheating of the assay tubes during sterilization may produce unsatisfactory results.

[184] György: *Nature*, **133**, 498 (1934).

It should be noted that the term vitamin B_6 includes not only pyridoxine but pyridoxal and pyridoxamine as well, all three compounds being found in nature.

This is the reason for the anomalous results obtained in microbiological[185] and chemical assays[186] for vitamin B_6 which led to the discovery of the aldehyde and amine derivatives, pyridoxal and pyridoxamine. These derivatives have biological activity equal to that of pyridoxine for rats and somewhat less for chicks, but their utilization by microorganisms varies considerably. All three compounds are equally active for *Saccharomyces carlsbergensis*, while pyridoxal and pyridoxamine are less active than pyridoxine for *Saccharomyces cervisiae;* on the other hand the aldehyde and amine are relatively more active for *L. helveticus* or *S. fecalis* R, and only pyridoxal has activity for *L. casei*. Evidence suggests the existence of members of the vitamin B_6 complex as yet unknown.[187] It is probable that these are known forms bound in such a way that they are not released by conventional methods of hydrolysis.

Physiological and Clinical Aspects of Pyridoxine. Deficiency of vitamin B_6 in rats causes acrodynia (see Fig. 279), edema, inhibition of growth, and nerve degeneration. The vitamin is essential for the chick, rat, dog, and pig, and for numerous microorganisms. The significance of the vitamin is claimed to be related in part to the metabolism of unsaturated fatty acids. The dermatitis observed in vitamin B_6 deficiency closely resembles that noted in cases of fatty acid deficiency. Some observers have reported that the addition of certain unsaturated fatty acids to a diet deficient in vitamin B_6 protects against the appearance of the deficiency syndrome of the vitamin.

The role of pyridoxine in amino acid metabolism is illustrated by the increased severity of symptoms upon the addition to a pyridoxine-poor diet of supplemental levels of cystine, methionine, tryptophan, glycine, or serine. Pyridoxine-deficient infants[188] or rats[189] lose their normal capacity to form niacin and N^1-methylnicotinamide from tryptophan. However the dependence of the tryptophan conversion on pyridoxine is questioned by some investigators.[190] Vitamin B_6 deficiency in rats and swine is characterized by the appearance in the urine of xanthurenic acid, a metabolite of tryptophan. All three known members of the vitamin B_6 group—pyridoxine, pyridoxal, and pyridoxamine—are converted by organisms capable of utilizing them into amino acid decarboxylases.[191] Pyridoxal phosphate functions as a coenzyme which, when combined with the apoenzyme (the protein moiety of the enzyme), such as is present in *Streptococcus fecalis* R grown in a medium deficient in vitamin B_6, catalyzes decarboxylation reactions. Amino acids reported to require pyridoxal phosphate as a coenzyme for decarboxylation include tyrosine, lysine, arginine, ornithine, glutamic acid, and dioxyphenylalanine ("dopa"), the end products being, respectively, tyramine, cadaverine, agmatine, putrescine, γ-aminobutyric acid, and 3,4-dihydroxyphenylethylamine.

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[185] Snell: *J. Biol. Chem.*, **157**, 491 (1945).

[186] Hochberg, Melnick, and Oser: *J. Biol. Chem.*, **154**, 313 (1944); **155**, 119 (1944).

[187] Melnick, Hochberg, Himes, and Oser: *J. Biol. Chem.*, **160**, 1 (1945).

[188] Synderman, Holt, Carretero, and Jacobs: *J. Clin. Nutrition*, **1**, 200 (1953).

[189] Schweigert and Pearson: *J. Biol. Chem.*, **168**, 555 (1947).

[190] Sarett: *J. Biol. Chem.*, **182**, 691 (1950).
Heimberg, Rosen, Leder, and Perlzweig: *Arch. Biochem.*, **28**, 225 (1950).

[191] Gunsalus and Bellamy: *J. Biol. Chem.*, **155**, 357 (1944); Bellamy, Umbreit, and Gunsalus: *J. Biol. Chem.*, **160**, 461 (1945); O'Kane and Gunsalus: *J. Biol. Chem.*, **170**, 425, 433 (1947).

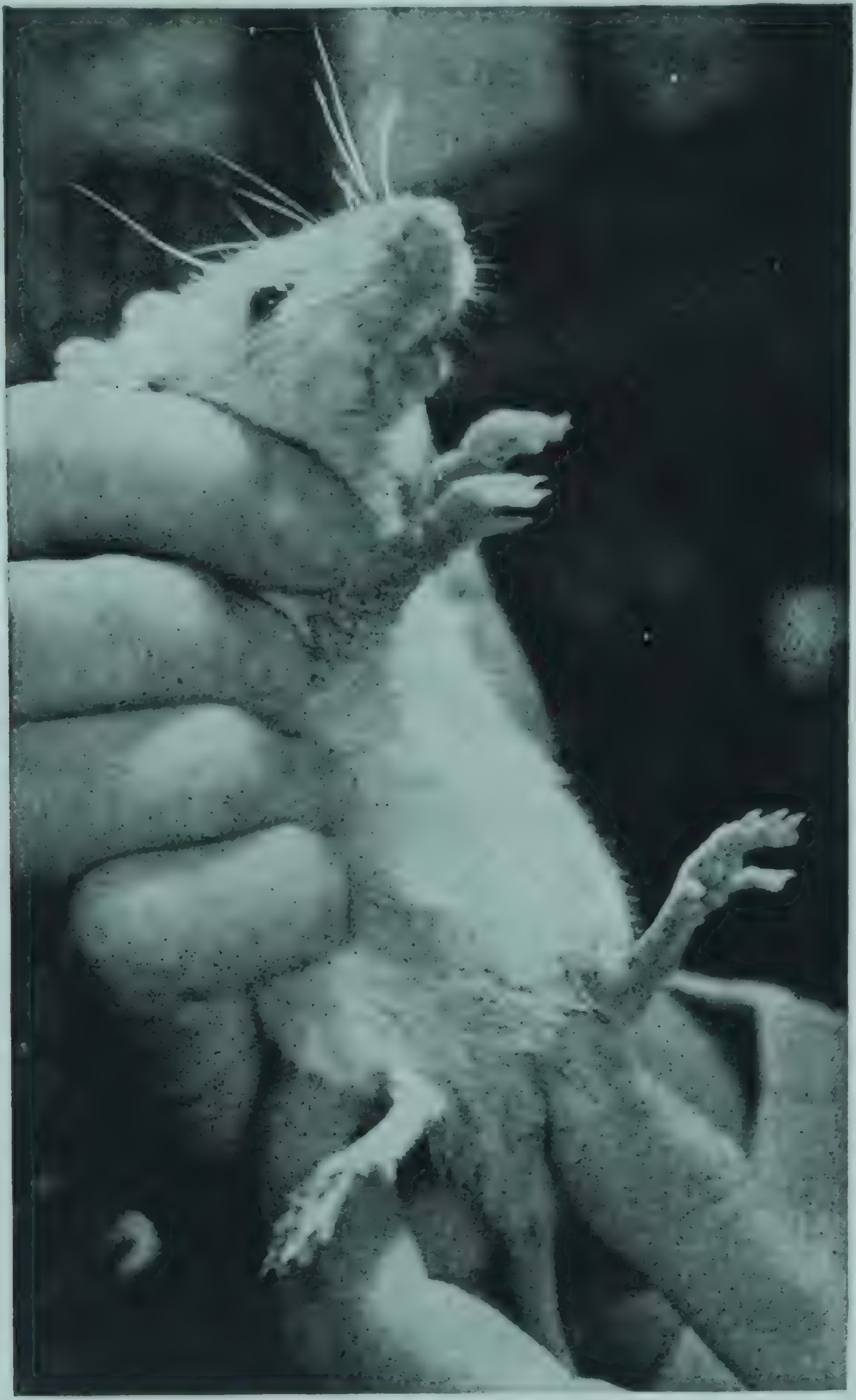

FIG. 279. Pyridoxine-deficient rat.
Note edema and dermatitis of paws and nose.

The high specificity of decarboxylation reactions permits their use for analytical purposes. Glutamic acid decarboxylase is present in the brain tissue of rats, and in vitamin B_6-deficiency it is the pyridoxal phosphate coenzyme that is depleted rather than the apoenzyme.[192]

The same coenzyme, pyridoxal phosphate, has been demonstrated to function in transamination reactions[193] of which the following is an

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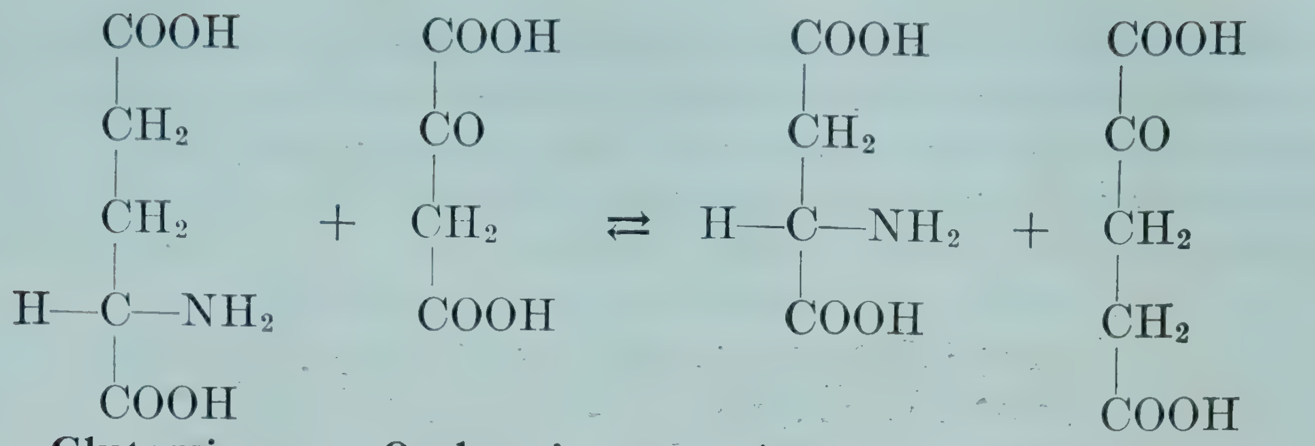
[192] For studies of this glutamic acid decarboxylase, see papers by Awapara, *et al.*; Udenfriend; and Roberts, *et al.* in *J. Biol. Chem.*, 1950-51.

[193] Lichstein, Gunsalus, and Umbreit: *J. Biol. Chem.*, **161**, 311 (1945).



Glutamic acid **Oxalacetic acid** **Aspartic acid** **α-Ketoglutaric acid**

example. Such reactions are suggested as the explanation of the growth response of rats to keto and hydroxy analogs of certain amino acids.[194] Evidence also indicates that vitamin B_6 participates in enzyme systems involved in the synthesis of amino acids by microorganisms.

The fact that pyridoxine derivatives play a part in such a variety of amino acid reactions would seem to justify the belief that vitamin B_6 is required in the nutrition of man, as it is for rodents, fowl, and other species. While no clear-cut deficiency syndrome is seen clinically which is comparable to thiamine or niacin deficiency, vitamin B_6 deficiency has been produced experimentally in man.[195] The daily human requirement of vitamin B_6, estimated chiefly on the basis of animal experiments, is 2 mg. The vitamin B_6 compounds are excreted mainly as 4-pyridoxic acid.

Pyridoxine has been used clinically in acne and other dermatologic disorders, but its value has yet to be conclusively demonstrated. Parkinson's disease, muscular dystrophy, and especially nausea and vomiting of pregnancy are among the conditions which have been treated with pyridoxine, but the results are too indefinite to justify associating these conditions with vitamin deficiencies. Clinical vitamin deficiencies, particularly of the B group, generally are of a multiple nature. Pellagrins who responded only partially to the administration of thiamine, riboflavin, and niacin showed considerable improvement after intravenous administration of pyridoxine.

Storage and Synthesis of Vitamin B_6. Vitamin B_6 is present in most animal tissues, with high concentrations in the liver. It is synthesized by bacteria in the rumen of sheep and cattle.

Distribution of Vitamin B_6.[196] In natural materials vitamin B_6 occurs principally bound to proteins. Rich sources are yeast and rice polishings. Seeds and cereals are good sources, especially the germ. In rice bran the vitamin is present to a small extent in the free form, but a major portion is a bound complex of pyridoxine, readily hydrolyzable by heating with strong acids. Pyridoxine was isolated from rice polishings in 1932 by Ohdake before its identity as a vitamin was known. In yeast and liver, it is present chiefly as bound pyridoxamine, though small concentrations of pyridoxine and pyridoxal are also found.

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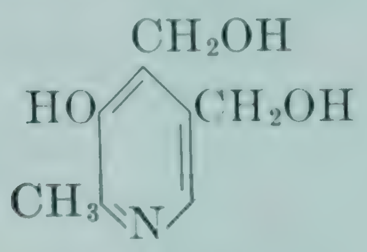
[194] Meister and White: *J. Biol. Chem.*, **191**, 211 (1951); Holden, Wildman, and Snell; *Ibid.*, **191**, 559 (1951).

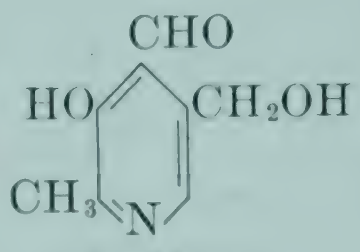
[195] Mueller and Vilter: *J. Clin. Invest.*, **29**, 193 (1950).

[196] See Appendix IV.

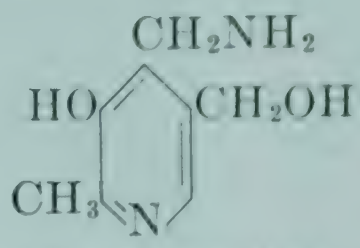
Chemistry of Vitamin B_6. Pyridoxine hydrochloride is a white, odorless, slightly bitter, crystalline solid, melting at 207° C. with slight decomposition. It is optically inactive and very soluble in water, slightly soluble in 95 per cent alcohol and in acetone, and insoluble in ether. The free base is also a colorless, crystalline solid, melting at 160° C. It is soluble in water, acetone, and alcohol, and slightly soluble in ether and chloroform. The aqueous solution of pyridoxine hydrochloride has a pH of approximately 3. It is adsorbed from acid solution on zeolite, charcoal, or fuller's earth, and may be eluted from zeolite with 10 per cent potassium chloride or from fuller's earth with a weak alkali. The structures of pyridoxine (2-methyl-3-hydroxy-4,5bis(hydroxymethyl)pyridine), pyridoxal, and pyridoxamine are:



Pyridoxine



Pyridoxal



Pyridoxamine

Pyridoxine, pyridoxamine, and pyridoxal can be separated by paper chromatography and distinguished by their relative positions on the strip. For this purpose a "bioautographic" procedure developed in the senior authors' laboratory[197] is used. The developed strip is laid flat on an agar plate containing a B_6-deficient medium and lightly seeded with *Saccharomyces carlsbergensis*, an organism capable of utilizing any of these forms of the vitamin. After incubation, the loci of B_6-factors on the strip are recognized by the zones of yeast growth.

Early chemical and microbiological studies of vitamin B_6 were complicated by the presence of the several forms, and by the facility of transformation of one compound to another. Conditions favoring amination or partial oxidation of pyridoxine result in the formation of some of the amine or aldehyde. This is attended by considerable change in the capacity for growth stimulation of certain microorganisms.

The three known forms of vitamin B_6 possess characteristic absorption curves in the ultraviolet region of the spectrum. These curves show no absorption maximum common to all three compounds. However, the total concentration of vitamin B_6 present in a solution relatively free of interfering substances may be determined by measurement at 325 mμ and pH 6.75. Under these conditions, the $E^{1\%}_{1\text{ cm.}}$ value for each of the compounds is 440.[186]

Pyridoxine, pyridoxal, and pyridoxamine are destroyed by exposure to light. Destruction is most rapid in the ultraviolet region of the spectrum in neutral or alkaline solution. Though all three compounds are comparatively stable to light in 0.1 N acid, pyridoxamine is slightly sensitive. The photolysis of vitamin B_6 is not affected by the presence of air. All three forms of the vitamin are stable to heating at 100° C. with 5 N sulfuric or hydrochloric acid; only pyridoxal is unstable to heat in alkaline solutions. All three compounds are stable to mild oxidizing agents like manganese dioxide in alkaline solution or hydrogen peroxide in neutral solution at room temperature. However, they are destroyed by potassium permanganate or by manganese dioxide in acid solution at room temperature, or by heating with nitric acid, potassium permanganate, or hydrogen peroxide.

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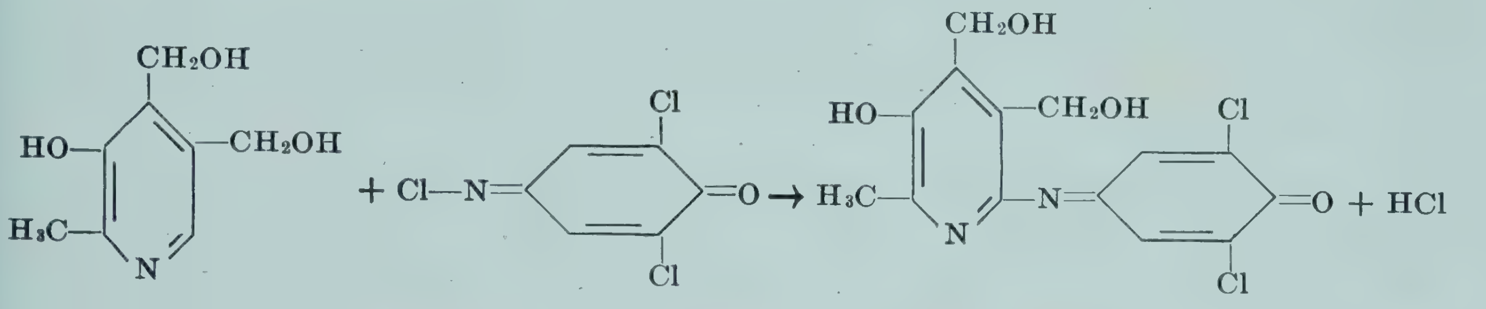
[197] Winsten and Eigen: *Proc. Soc. Exp. Biol. Med.*, **67**, 513 (1948).

DETERMINATION OF VITAMIN B_6

Since a major proportion of the vitamin B_6 present in natural materials occurs in bound forms, assays must be preceded by hydrolysis by enzymes or by heating with acid. Pyridoxine reacts with ferric chloride to produce a compound having an orange-red color. Pyridoxine is phenolic and reacts with diazonium salts to produce dyes. The other forms probably have similar reactions. Pyridoxine, pyridoxal, and pyridoxamine couple with 2,6-dichloroquinone chloroimide to produce a blue dye, the last two being about half as reactive as the first. The reaction with pyridoxine is shown in the following equation. In the presence of boric acid, pyridoxine alone forms a stable complex which does not couple with the reagent, thus permitting its differentiation from pyridoxal and pyridoxamine in pure systems.[186] The low values for the pyridoxine content of natural materials obtained by this procedure indicated the existence of other, possibly conjugated, forms of vitamin B_6. At any rate it is not recommended as an analytical method except in relatively pure mixtures where pyridoxine is the predominant B_6 factor, as in pharmaceutical preparations.



Pyridoxine **2,6-Dichloroquinone chloroimide** **Blue dye**

Microbiological methods for the determination of vitamin B_6 are based on the growth stimulation of yeast or bacteria. These are complicated by the variable responses of the microorganisms to the different forms of the vitamin. All three forms have equal activity on a molar basis for *Saccharomyces carlsbergensis.* For *Saccharomyces cerevisiae,* however, the amine has 40 per cent, and the aldehyde 46 per cent, of the activity of pyridoxine. Though pyridoxine itself is inactive for *Lactobacillus casei* and *Streptococcus fecalis* R, pyridoxal is active and pyridoxamine inactive for the former, whereas pyridoxamine is active and pyridoxal 36 per cent as active as pyridoxamine for the latter. Assuming that vitamin B_6 complex contains no other members than pyridoxine, pyridoxal, and pyridoxamine, differential assays may be conducted on natural materials or on mixtures of the three synthetic forms, employing several microorganisms. Such assays are difficult, however, because of the ease of conversion from one form to any other. Pyridoxal when autoclaved with hydrolyzed casein produces some pyridoxamine, resulting in increased activity for *S. fecalis.* The reverse of this transamination reaction occurs when pyridoxamine is autoclaved with α-ketoglutaric acid. Such treatment results in increased activity for *L. casei* and decreased activity for *S. fecalis.* Autoclaving pyridoxine with cystine, glycine, ammonia, or thioglycollic acid or treating with hydrogen peroxide results in the formation of "pseudopyridoxine" (probably pyridoxal and pyridoxamine), having increased biological activity for lactic acid organisms. Attempts have been made to prevent these transamination or partial oxidation reactions by sterilizing the hydrolyzed samples and the microbiological media separately, and then combining them aseptically. Such procedures, however, may not give a true picture of the concentrations of the various forms originally present in the test materials, since the undesirable conversions may occur during the sterilization of the samples themselves. When animal tissues are autoclaved with pyridoxine, the activity for certain lactic acid bacteria may increase several thousandfold. Moreover, the differential assays are based on the assumption that pyridoxine, pyridoxal, and pyridoxamine are the only existing forms of vitamin B_6.

Certain anomalous results obtained in the assay of natural materials for vitamin B_6 have been interpreted to indicate the presence of forms of the vitamin as yet unknown. The bound pyridoxine in rice bran concentrates is completely liberated by autoclaving with 2 N sulfuric acid, so that analysis of such hydrolyzates with *Saccharomyces carlsbergensis* gives values agreeing with those of the rat assay. Hydrolysis of yeast and liver samples by the same treatment, however, gives low values by the microbiological procedure. When the sample is hydrolyzed by heating with comparatively weak acid, 0.055 N sulfuric, the values agree with those found in the rat assay. The low results are not attributable to destruction of the known forms of vitamin B_6 by the strong acid treatment, since pyridoxine, pyridoxal, or pyridoxamine may be heated in the presence of yeast and liver without measurable destruction.

Since all members of the vitamin B_6 complex, both known and unknown, have equal activity for *Saccharomyces carlsbergensis* and for the rat, reliable microbiological assays of natural materials may be made employing this microorganism. As described above, the concentration of sulfuric acid required for the hydrolytic liberation of bound forms of the vitamin varies with the type of product under assay. Hence careful attention must be given to this preliminary step. To destroy thiamine which, under certain conditions, may interfere with the pyridoxine assay, it has been suggested that the sample be hydrolyzed with N sodium hydroxide under pressure.[198]

Biological procedures have been described for the estimation of vitamin B_6 based on the growth of rats and on the cure of specific rat dermatitis. Of these, the methods of Dimick and Schreffler[199] and of Sarma, Snell, and Elvehjem[200] have been widely used. The latter authors have also reported a chick-growth assay.

The principal urinary excretion product resulting from the ingestion of pyridoxine by man is pyridoxic acid (2-methyl-3-hydroxy-4-carboxy-5-

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[198] Morris, Herwig, and Jones: *Analyst*, **74**, 37 (1949).

[199] Dimick and Schreffler: *J. Nutrition*, **17**, 23 (1939).

[200] Sarma, Snell, and Elvehjem: *J. Biol. Chem.*, **165**, 55 (1946).

hydroxymethyl pyridine). This compound is converted by heating with strong acid to a highly fluorescent lactone which can be determined by simple fluorometric means.[201] This reaction is useful in metabolic studies of vitamin B_6.

Determination of the Vitamin B_6 Complex** (**Microbiological Method of Atkin, Schultz, Williams, and Frey**):*[202] ***Principle. The growth stimulation of a strain of the yeast *Saccharomyces carlsbergensis* by vitamin B_6 is employed for the microbiological assay of the vitamin.

Procedure: Preparation of Yeast Inoculum. **Prepare a fresh slant of Culture 4228 (American Type Culture Collection), a strain of *Saccharomyces carlsbergensis*, on "Difco" malt agar and incubate for 24 hours at 30° C. Remove a quantity of fresh growth with a sterile wire loop and suspend in 10 ml. sterile 0.9 per cent saline in a colorimeter tube. With the aid of a densitometer[203] or photoelectric colorimeter, adjust the concentration to an equivalent of 1 mg. moist yeast per ml. by adding sterile saline. Dilute 5 ml. of the adjusted suspension with 45 ml. saline in a sterile Erlenmeyer flask.**

Preparation of the Sample. **Suspend a portion of the sample containing between 2 and 4 μg. vitamin B_6 in 180 ml. 0.055 N sulfuric acid.[204] Autoclave at 15 pounds pressure for 1.5 hours, cool, neutralize to pH 5.2, and dilute to 200 ml. Centrifuge if turbid and assay the clear supernatant extract.**

Preparation of Basal Medium. **Mix 100 ml. sugar and salts solution,[205] 20 ml. potassium citrate buffer, 20 ml. casein hydrolyzate, 10 ml. thiamine solution, 10 ml. inositol solution, 4 ml. biotin solution, 5 ml. calcium pantothenate solution, and 10 ml. niacin solution. Dilute to 200 ml.**

Microbiological Assay. **Place 5 ml. basal medium in each of a series of 18-mm. pyrex test tubes. In successive tubes pipet respectively 0.25, 0.50, 1.00, 2.00, 3.00, and 4.00 ml. of extract. With each assay series also include a reference series consisting of tubes containing 0, 5, 10, 15, 20, 30, and 40 millimicrograms pyridoxine hydrochloride. Adjust the total volume in each tube to 9 ml. Plug the tubes and steam for 10 minutes and cool. Under aseptic**

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[201] Huff and Perlzweig: *J. Biol. Chem.*, **155**, 345 (1944).

[202] Atkin, Schultz, Williams, and Frey: *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943). The addition of niacin to the basal medium was proposed by Hopkins and Pennington: *Biochem. J.*, **41**, 110 (1947).

[203] Calibrate the densitometer with a suspension of moist baker's yeast.

[204] Some materials, wheat, and wheat products require more acid. For such samples use 2.0 N sulfuric acid.

[205] *Sugar and Salts Solution.* Dissolve 200 g. C.P. dextrose (anhydrous), 2.2 g. monopotassium phosphate, 1.7 g. potassium chloride, 0.5 g. calcium chloride dihydrate, 0.5 g. magnesium sulfate, 0.01 g. ferric chloride, and 0.01 g. manganese sulfate in distilled water and dilute to 1000 ml.

Potassium Citrate Buffer. Dissolve 100 g. potassium citrate monohydrate and 20 g. citric acid monohydrate in distilled water and dilute to 1 liter.

Casein Hydrolyzate Solution. Neutralize 80 ml. "vitamin-free" casein hydrolyzate (10 per cent solution, obtainable from General Biochemicals, Inc., Chagrin Falls, O.) to pH 4–6 and dilute to 100 ml.

Thiamine Solution. 10 μg. per ml.

Inositol Solution. 1 mg. per ml.

Biotin Solution. 0.8 μg. per ml.

Calcium Pantothenate Solution. 200 μg. per ml.

Niacin Solution. 0.1 mg. per ml.

conditions introduce into each tube 1 ml. of the yeast inoculum. Place the tubes in a mechanical shaker for 16–18 hours at 30° C. Immediately thereafter estimate the yeast growth turbidimetrically in a densitometer or photoelectric colorimeter with a 660 or 720 mμ filter.[206]

CALCULATION. Plot the results of the reference series in per cent absorption against mμg. of pyridoxine hydrochloride. Estimate the values for the unknowns from the graph. Calculate the pyridoxine content of the sample for each tube. Average all values which agree within 10 per cent of their mean.

Interpretation. The vitamin B_6 group consists of pyridoxine, pyridoxal, pyridoxamine, and one or more unidentified labile factors. All members of the complex show comparable activity for *Saccharomyces carlsbergensis* (Culture 4228) and the rat. Hence this procedure measures biological vitamin B_6 activity rather than the concentration of pyridoxine or any single derivative.

PANTOTHENIC ACID

Bios, a growth stimulant essential for yeast, was described in 1901 by Wildiers.[207] Numerous attempts were made to elucidate the nature of this factor (or, as later discovered, factors), but not until 1933 was a crystalline product isolated by R. J. Williams and his co-workers,[208] who called it *pantothenic acid* (and later suggested *pantothen*) to indicate its universal distribution. In the meantime, other groups of workers were interested in an extract prepared from liver, variously designated "filtrate factor," "chick antidermatitis factor," or Factor II, which was found to be necessary to restore growth and prevent a severe dermatitis in chicks receiving a diet of heated grains. The introduction of microbiological assays using lactic acid bacteria stimulated progress along these lines which were merged in 1939. Jukes,[209] testing potent concentrates of Williams' yeast-growth factor on chicks, showed it to be identical with the "filtrate factor," and Woolley, Waisman, and Elvehjem[210] showed that it was a derivative of β-alanine which Williams had reported to be a cleavage product of pantothenic acid.

The crystallization of pantothenic acid, its structure, and finally its synthesis were reported in 1940 by the Merck group of investigators.[211] Pantothenic acid is available commercially as the calcium and sodium salts.

Physiological and Clinical Aspects of Pantothenic Acid. The wide distribution of pantothenic acid in animal and plant tissues gave indication that it plays a fundamental role in metabolism. The nature of its function has become clear since the discovery by Lipmann and associates[212] of coenzyme A, of which pantothenic acid is a molecular component. It is now believed that coenzyme A is the functional form of pantothenic acid in plant and animal tissues and in microorganisms.

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[206] In almost every case pigments, when present in the test extracts, absorb light maximally in the visible spectrum at about 400 to 450 mμ, but not to any extent in the range 660 to 720 mμ. Thus with the use of a proper filter no interference results from the increasing pigmentation of the solution with increasing quantities of test extract. In any case it is recommended that a separate tube containing 4.00 ml. test extract plus 1 ml. of water (no inoculum) be included in the series. Readings of this solution allow proportional corrections of the photometric densities of the serial tubes for absorption due to interfering pigments.

[207] Wildiers: *La Cellule*, **18**, 313 (1901).

[208] Williams, *et al.*: *J. Am. Chem. Soc.*, **60**, 2719 (1938).

[209] Jukes: *J. Am. Chem. Soc.*, **61**, 975 (1939).

[210] Woolley, Waisman, and Elvehjem: *J. Am. Chem. Soc.*, **61**, 977 (1939).

[211] Stiller, Harris, Finkelstein, Keresztesy, and Folkers: *J. Am. Chem. Soc.*, **62**, 1785 (1940).

Pantothenic acid is required by the chick, rat, pig, dog, and other vertebrates. Deficiency symptoms are quite varied in different species. In the chick, the syndrome includes keratitis, dermatitis, fatty liver, lesions of the spinal cord, and involution of the thymus; the vitamin is necessary for reproduction in hens but not for egg production. In rats, insufficient intake of dietary pantothenic acid results in necrotic lesions of the adrenal cortex and other related symptoms. Regulation of salt and water balance are functions of the adrenal cortex. Pantothenic acid deficiency results in increased appetite for salt, and a low-salt diet promotes the graying of hair which results from pantothenic acid deficiency. Deprivation of water produces "bloody whiskers" (i.e., porphyrin staining) in rats, a condition also brought about by pantothenic acid deficiency.

Achromotrichia or graying of hair in black rats ("rusting" in white rats) on diets lacking pantothenic acid, and similar phenomena in dogs and foxes, probably are due to a complex deficiency, since pantothenic acid itself is less effective than extracts containing mixtures of B-complex factors. Pantothenic acid has not been established to be of specific value in restoring the color to human gray hair.

The "spectacled eye" condition in rats, associated with a deficiency of inositol or biotin, may develop in the absence of sufficient pantothenic acid. Pantothenic acid is required in the metabolism of all bacteria. Those which do not require an external supply synthesize it themselves. Dietary deficiency of this factor may result in secondary vitamin deficiencies due to diminished synthesis by intestinal flora.

The physiologically active form of pantothenic acid in the animal organism appears to be coenzyme A. This coenzyme (abbreviated CoA) participates in a wide variety of reactions involving the transfer of acetate or two-carbon units. Its acetyl derivative (acetyl CoA)

$$\text{CoA} + \text{acetate} \xrightleftharpoons{\text{ATP}} \underset{\textbf{Acetyl CoA}}{\text{CoA}\cdot\text{COCH}_3}$$

is considered to be synonymous with "active acetate" and has been described as the "pivot on which acetyl transfer mechanisms turn."[213] However, it is necessary to point out that many of these metabolic transformations are quite complex and involve the mediation of numerous enzymes and coenzymes. CoA participates in the acetylation of amines; examples are sulfanilamide, glycine, etc. In these reactions the carbonyl group of the acetyl radical is involved:

$$\text{CoA}\cdot\text{C}^*\text{OCH}_3 + \text{R}\cdot\text{NH}_2 \rightarrow \text{R}\cdot\text{NH}\cdot\text{C}^*\text{OCH}_3 + \text{CoA}$$

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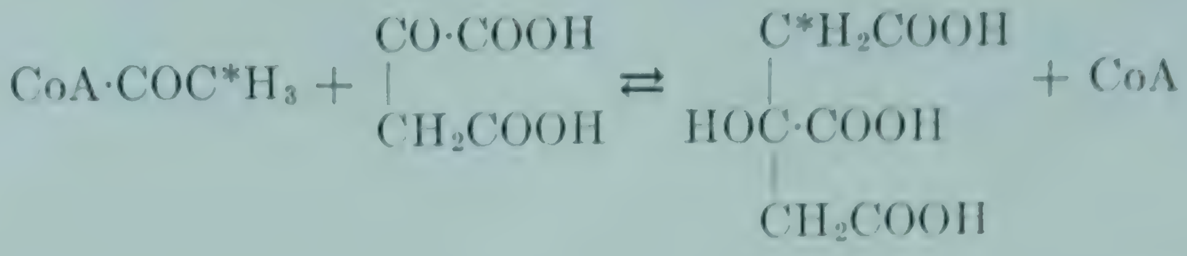
[212] Lipmann, Kaplan, Novelli, Tuttle, and Guirard: *J. Biol. Chem.*, **167**, 869 (1947); **186**, 235 (1950).

[213] Welch and Nichol: *Ann. Rev. Biochem.*, **21**, 633 (1952).

In contrast with this mechanism, the methyl group is involved in CoA condensation reactions such as the formation of citric from oxalacetic acid:



$$\mathrm{CoA{\cdot}COC^*H_3} + \begin{array}{l}\mathrm{CO{\cdot}COOH}\\ |\\ \mathrm{CH_2COOH}\end{array} \rightleftarrows \begin{array}{c}\mathrm{C^*H_2COOH}\\ |\\ \mathrm{HOC{\cdot}COOH}\\ |\\ \mathrm{CH_2COOH}\end{array} + \mathrm{CoA}$$

a reaction mediated by a condensing enzyme.[214]

It is believed that all substances (e.g., acetaldehyde, acetate) entering the tricarboxylic acid cycle as two-carbon fragments generate acetyl CoA.[215] This may be illustrated by the following reactions for acetate and pyruvate:

$$\begin{aligned}\text{CoA} + \text{acetate} &\overset{\text{ATP}}{\rightleftharpoons} \text{Acetyl CoA}\\ \text{CoA} + \text{pyruvate} &\rightleftharpoons \mathrm{CO_2} + \text{Acetyl CoA}\end{aligned}$$

The foregoing indicates, in part at least, the vital role played by the pantothenic acid coenzyme in carbohydrate metabolism. Acetyl transfer also occurs in the metabolism of fatty acids and β-keto fatty acids. *In vitro* studies on butyrate oxidation[216] have suggested that for every two carbons of a fatty acid chain one mole of acetyl CoA is generated; in a secondary reaction acetyl phosphate is formed releasing CoA for further degradation of the chain. It has furthermore been demonstrated that two moles of acetyl phosphate can be condensed to form acetoacetate. What relation these facts have to the biosynthesis of fatty acid is not yet established, since labeled acetoacetic acid is not incorporated into fatty acid in rat liver preparations, although labeled cholesterol is formed.

The role of two-carbon fragments in intermediary metabolism is under active investigation, and reference should be made to Chapter 33 and to the current literature (see Bibliography, p. 1293). Much of the work in this field is based on *in vitro* studies of microorganisms, tissue slices, and extracts thereof, and its significance for the living animal awaits further study.

The bound forms of pantothenic acid in natural materials are available to higher animals but not to microorganisms. For the latter, it must first be released by suitable hydrolytic procedures. Certain synthetic forms of bound pantothenate have been investigated. Ethyl monoacetylpantothenate is active for the rat and chick, and ethyl pantothenate for the rat. However, neither of these forms shows activity for microorganisms unless they are previously hydrolyzed by procedures similar to those employed for the release of bound pantothenate in natural materials.

Certain structurally related compounds behave as antivitamins toward pantothenic acid. Pantoyltaurine (the sulfonic analog), pantoyltauramine, and homopantoyltaurine inhibit the growth of bacteria which require an external source of the vitamin. These antivitamins probably operate by competing in some enzyme systems. One observer has found that the administration of pantoyltaurine to mice produces symptoms similar to those of pantothenic acid deficiency. This observation could not be confirmed by other investigators. For a further discussion of antivitamins, see Chapter 36.

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[214] Ochoa, Stern, and Schneider: *J. Biol. Chem.*, **193**, 691 (1951).

[215] Ochoa and Stern: *Ann. Rev. Biochem.*, **21**, 547 (1952).

[216] Barker, in *Phosphorus Metabolism*, Baltimore, Johns Hopkins University Press, 1951.

Possibly because of intestinal synthesis, pantothenic acid deficiency has not been observed in man and hence its importance in human nutrition has not been clarified. The daily requirement, as estimated from the analysis of good diets and from urinary excretion data is approximately 5–10 mg. per 2500 calories. Pantothenic acid is present in normal human blood or plasma to the extent of 20 to 40 μg. per 100 ml.

The physiological effect of pantothenic acid is highly specific for that molecule. Replacement of the β-alanine portion with α-alanine, or with other amino acids having structures similar to β-alanine, produces inactive compounds. The optical antipode of the natural L-isomer is also inactive. The β-alanine portion alone is sufficient for some yeasts and diphtheria bacilli, and is partly available for rats, but not for chicks. These organisms probably utilize the β-alanine for the synthesis of pantothenic acid. Certain hemolytic bacteria require only the dihydroxydimethylbutyryl portion of the molecule. Hydroxypantothenic acid has a biological activity varying from 2 to 25 per cent of that of pantothenic acid, depending upon the organism employed and the conditions of assay.

Storage and Synthesis of Pantothenic Acid. Pantothenic acid from external sources is not required by sheep and cattle, since the vitamin is synthesized by microorganisms in the rumen of these animals. It may be synthesized by certain molds, bacteria, and yeast, when grown in media devoid of pantothenic acid. Some yeasts require an external supply of β-alanine. Pantothenic acid is produced by green plants after they have developed sufficiently to perform their photosynthetic functions.

Distribution of Pantothenic Acid. Pantothenic acid is present in all living tissues. Excellent sources are liver, kidney, rice bran, molasses, egg yolk, peanuts, and peas. Appreciable concentrations are also found in sweet potatoes, oats, wheat, rye, barley, and broccoli. Pantothenic acid occurs in natural materials both free and combined, though a major fraction of the vitamin in animal sources is bound. While it has not been definitely established that all forms of conjugated pantothenic acid released by an enzymatic digestion procedure are equally available for animal species, rat-growth studies indicate this to be so for coenzyme A and *L. bulgaricus* factor. In certain foods or extracts (liver powder, yeast, wheat-bran and rice-bran extracts) where rat assays were reported to be considerably higher than microbiological assays, the discrepancies may have been due to unidentified dietary factors or incomplete enzymatic release of pantothenic acid prior to microbiological assay.

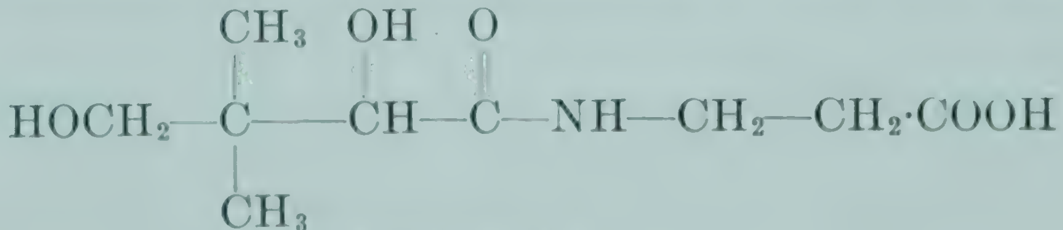
Chemistry of Pantothenic Acid. Pantothenic acid (D-N-(α,γ-dihydroxy-β,β,-dimethylbutyryl)-β-alanine) is a condensation product of β-alanine and a hydroxyl- and methyl-substituted butyric acid. It is a pale yellow, unstable, viscous oil.

The natural or vitamin-active form of pantothenic acid is the dextrorotatory isomer, specific rotation $[\alpha]_D^{25} = +37.5°$. Synthetic pantothenic acid is produced commercially as the calcium or sodium salts, in either dextrorotatory or racemic form, the latter having half the potency of the former. The dextrorotatory alcohol corresponding to pantothenic acid, pantothenyl alcohol, is vitamin-active and is also available commercially. It is claimed to be more stable than the acid at pH below 6. Pantothenic acid is adsorbed by "Norit" at pH 3.5 or by aluminum oxide previously activated with hydrochloric acid but not by fuller's earth. It may be eluted from "Norit" with ammonia or pyridine and methanol. Pantothenic acid is soluble in water, ethyl acetate, dioxane, and glacial acetic acid, and is slightly soluble in ether and amyl alcohol, and insoluble in chloroform and benzene. It is unstable to heat in the dry state or in acid or alkaline medium. In the pH range 5 to 7 it may be autoclaved at 15 pounds pressure for one-half hour without loss.

PANTOTHENIC ACID CONTENT OF FOODS
(AFTER NEILANDS AND STRONG*)

Food	*Pantothenic Acid mg. per 100 g.*
Alfalfa	6.2
Beef, dried	5.6
Egg, fresh	5.5
Liver powder	39.3
Mackerel	1.4
Salmon	2.6
Sardines	1.5
Spinach	2.7
Tuna	1.4
Wheat germ meal	2.9
Yeast, dried	44.3

* Neilands and Strong: *Arch. Biochem.*, **19**, 287 (1948). Based on microbiological assay after digestion with liver enzyme and phosphatase.

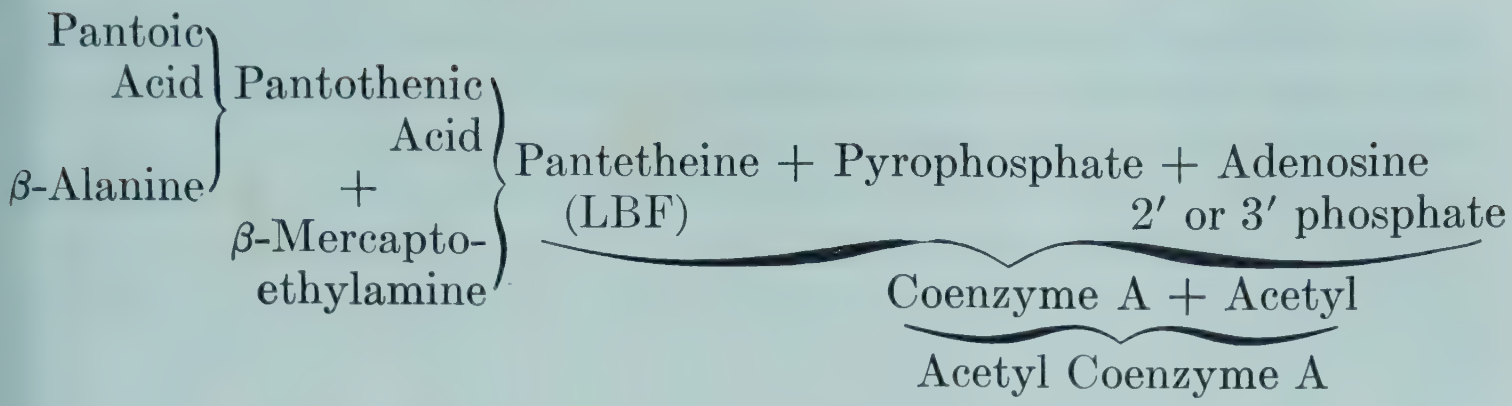


$$HOCH_2-\underset{\displaystyle CH_3}{\underset{|}{\overset{\displaystyle CH_3}{\overset{|}{C}}}}-\overset{\displaystyle OH}{\overset{|}{CH}}-\overset{\displaystyle O}{\overset{\|}{C}}-NH-CH_2-CH_2\cdot COOH$$

Pantothenic acid

The butyrolactone moiety of pantothenic acid is known as pantolactone and the corresponding acid is pantoic acid, $HOCH_2C(CH_3)_2CHOH-COOH$. A naturally occurring growth factor for certain microorganisms (*Lactobacillus bulgaris* factor or LBF) has been identified as a β-mercaptoethylamine derivative of pantothenic acid and given the name pantetheine.

The corresponding disulfide, pantethine, exists presumably in equilibrium with the reduced form. The following diagram illustrates the relationship between these compounds in terms of the various moieties in the molecule of acetyl coenzyme A.



DETERMINATION OF PANTOTHENIC ACID

No satisfactory chemical method is available for the determination of pantothenic acid in natural products, although several procedures have been described for pharmaceutical preparations. One[217] is based on the hydrolytic release of the β-alanine moiety, followed by colorimetric estimation as the alkaline 2,4-dinitrophenylhydrazone. Interfering B vitamins may be removed chromatographically, but ascorbic acid still presents a problem.

Another procedure[218] is based on a reaction between the pantoyl lactone moiety and hydroxylamine in alkaline solution and the development of a purple color upon acidification and addition of ferric chloride. In contrast to the previous method, this may be applied to the estimation of pantothenyl alcohol, known commercially as "Panthenol." However interference due to ascorbic acid, pantoic acid, and the lactone must be eliminated by adsorption on a basic resin XE-75.[219]

Determination of Pantothenic Acid (Microbiological). The absolute requirement of a number of microorganisms for free pantothenic acid was primarily responsible for the original discovery of this vitamin. Many determinations of the pantothenic acid content of natural materials have been published. By far the majority of these have been microbiological procedures although a considerable amount of work has also been reported based on the use of chicks,[220] rats, and other species as test animals. The repeated observation that animal assays in general gave higher values than the microbial tests, together with several early reports on the release of additional increments of pantothenic acid activity by autolysis of tissues or treatment with specific enzyme preparations, led to the recognition of "bound forms" of the vitamin which occurred in many natural materials. However, the release of activity from these hypothetical conjugates was neither striking nor consistent.

The discovery that coenzyme A was in reality a pantothenate-containing prosthetic group of enzymes responsible for transacetylation in living cells proved to be the key to the problem of bound or conjugated forms of the vitamin which had been shown to be unavailable to microorganisms such as *L. arabinosus.* A specially prepared enzyme obtained from pigeon or chicken liver was found capable of inactivating coenzyme A with the concurrent liberation of free pantothenate. At the same time, an alkaline phosphatase from intestine was shown to split CoA in another manner with the liberation of phosphate.[221]

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[217] Szalkowski, Mader, and Frediani: *Anal. Chem.*, **22**, 369 (1950).

[218] Wollish and Schmall: *Anal. Chem.*, **22**, 1033 (1950).

[219] György, Rubin, and de Ritter, in *Vitamin Methods*, Vol. 2, New York, Academic Press, Inc., 1951. This resin is available at Resinous Products Division, Rohm and Haas Co., Philadelphia 5, Pa.

[220] Jukes: *J. Biol. Chem.*, **117**, 11 (1937); *J. Nutrition*, **21**, 193 (1941).

By combining in a single treatment both an intestinal phosphatase and the CoA-splitting enzyme from pigeon liver, Neilands and Strong[222] showed that some natural materials contained as much as four times the amount of pantothenate which could be demonstrated by simple direct assay. They also showed that other nonspecific phosphatases were relatively ineffective in liberating the vitamin. On the basis of this work, it is evident that a large part of the data previously accumulated on the distribution of pantothenate in foods and tissues is in error and needs reinvestigation. Some of the new values reported are given in the table, p. 1190.

Determination of Pantothenic Acid: Modified Microbiological Method of Skeggs and Wright.[223] Pantothenic acid is determined by measurement of the growth stimulation of *Lactobacillus arabinosus* 17-5 by titration of the lactic acid formed or by turbidimetric determination of the cell population. The bound vitamin is first liberated by digestion with alkaline phosphatase and chicken liver enzyme.

Procedure: Preparation of liver enzyme.[224] **Liver from freshly killed chicken or pigeon is chilled, minced, and homogenized with 20 volumes of ice-cold acetone in a Waring blendor. The acetone-insoluble material is filtered and washed with acetone and ether and dried quickly in a current of dry air. The resulting liver powder[225] may be preserved in small, well-sealed vials under refrigeration for a considerable period. For use, the acetone powder is rubbed in a chilled mortar to a smooth paste with 10 times its weight of cold 0.02 M sodium bicarbonate solution. The suspension is centrifuged below 5° C. for 30 minutes at 3,000 r.p.m., and the reddish-brown supernatant liquid is preserved by freezing and holding below −20° C.**

This solution possesses good activity on coenzyme A but has the disadvantage of containing considerable amounts of pantothenic acid in a free form. Its use in the assay of materials of low pantothenate content presents the problem of an excessive blank. The amount of free vitamin may be substantially reduced by treatment of the solution with an anion-exchange resin.[226] The following procedure is recommended.

Prepare the resin for use by washing twice with 10 volumes of N HCl and centrifuging. The acid-treated resin is then washed 8 to 10 times with 10 volumes of water until the pH of the wash is about 5. The resin is left in a slurry of a consistency which can be pipetted with a 10 ml. serological pipet. To 1 volume of resin slurry is added a few drops of M tris-(hydroxymethyl)-aminomethane buffer, pH 8.3, to bring the pH to 8.0, and 1 volume of ice-cold liver enzyme solution is added and the suspension stirred for 3 to 5 minutes in an ice bath. The suspension is centrifuged at 5,000 r.p.m. The enzyme solu-

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[221] Lipmann, Kaplan, Novelli, Tuttle, and Guirard: *J. Biol. Chem.*, **167**, 869 (1947); Novelli, *et al.*: *J. Biol. Chem.*, **177**, 97 (1949); **192**, 181 (1951).

[222] Neilands and Strong: *Arch. Biochem.*, **19**, 287 (1948).

[223] Skeggs and Wright: *J. Biol. Chem.*, **156**, 21 (1944). A similar method has been adopted by the Association of Official Agricultural Chemists, *J. Assoc. Official Agr. Chemists*, **35**, 103 (1952).

[224] Novelli and Schmetz: *J. Biol. Chem.*, **192**, 181 (1951).

[225] The prepared acetone powder may be obtained from Armour and Co., Chicago, Ill.

[226] Dowex-1, 200-400 mesh, obtainable from the Dow Chemical Co., Midland, Michigan.

tion is again treated with another volume of anion resin slurry and centrifuged. This process usually reduces the free pantothenate content of the solution to less than one-tenth its original value. Preserve the preparation at −20° C. until ready for use.

Preparation of Intestinal Phosphatase. **This preparation can be obtained by following the procedure of Schmidt and Thannhauser[227] but is more conveniently obtainable from commercial sources.[228] A 2 per cent solution of this material usually contains about 100 Schmidt-Thannhauser units per ml. and is quite low in free pantothenic acid activity.**

Liberation of Bound Pantothenate. **To a sample containing 5 to 15 μg. bound pantothenate are added 0.1 ml. intestinal phosphatase, 0.2 ml. liver enzyme, 0.1 ml. M tris(hydroxymethyl)aminomethane buffer, pH 8.3, and the whole is made to a total volume of 1 ml. Suitable blank and control tubes should also be set up to allow a correction for any free pantothenate activity in the enzyme preparations. The tubes are incubated at 37° C. for 3 hours and then immersed in a boiling water bath to stop enzyme reactions. The samples are then diluted to bring them into the range of the usual standard curve employed in the pantothenic acid assay of Skeggs and Wright described below.**

Preparation of Medium. **Prepare the basal medium having the composition shown in the table below. Five liters may be prepared as a stock solution at one time provided the glucose and synthetic vitamins are omitted. This solution keeps indefinitely at room temperature under benzene even without sterilization. Prepare a stock vitamin solution containing 4 mg. each of thiamine, riboflavin, and niacin, 8 mg. pyridoxine hydrochloride, 0.4 mg. *p*-aminobenzoic acid and 10 μg. biotin per 100 ml. Store the vitamin supplement in a dark bottle in the refrigerator and renew monthly. Prepare a solution containing 100 μg. per ml. of calcium pantothenate.**

Culture. **Carry stab cultures[229] of *Lactobacillus arabinosus* 17-5 by monthly transfer in a medium containing 1 per cent yeast extract, 1 per cent glucose, and 1.5 per cent agar. After transfer, incubate the culture at 33° C. for 24 to 48 hours, then store in the refrigerator. Prepare the inoculum for the assay tubes by transferring from the stock culture to a sterile tube containing 10 ml. of the basal medium to which 0.2 μg. calcium pantothenate has been added. Incubate for 24 hours at 33° C., centrifuge, and discard the supernatant liquid. Resuspend the cells in 10 ml. physiological saline, centrifuge, and discard the supernatant. Resuspend the cells in sufficient physiological saline to produce a very light suspension.**

Assay. **Into a series of test tubes, pipet the following volumes: 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, and 5.00 ml. Similarly prepare a standard series of tubes from a dilution of the standard solution of calcium pantothenate containing 0.1 μg. per ml. Dilute the aliquots in both the standard and unknown series with distilled water so that each tube contains a total volume of 5 ml. To each tube add 5 ml. of the double strength medium, cover with cotton plugs or metal caps and heat in an autoclave at 15 pounds pres-**

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[227] Schmidt and Thannhauser: *J. Biol. Chem.*, **149**, 369 (1943).

[228] Intestinal Phosphatase (Calf), Armour and Company, Chicago, Ill.

[229] A culture of the organism may be obtained from the American Type Culture Collection, 2029 M Street, N. W., Washington 6, D. C., under the classification number 8014.

sure for 15 minutes. Add to each tube aseptically one drop of the inoculum prepared as described above. Incubate at 33° C. for 72 hours and determine the lactic acid produced by titration with 0.1 N sodium hydroxide, employing bromothymol blue as the indicator.

BASAL MEDIUM

Ingredient	*Per 100 ml. Medium (Double Strength)*	*Ingredient*	*Per 100 ml. Medium (Double Strength)*
Casein[230]	1.0 g.	Inorganic salts B (see p. 1160)	1.0 ml.
Cystine	20.0 mg.	Glucose	4.0 g.
Tryptophan	20.0 "	Thiamine chloride	200 μg.
Oleic acid	10.0 "	Riboflavin	200 "
Sodium acetate (anhydrous)	1.2 g.	Nicotinic acid	200 "
Adenine	1.0 mg.	Pyridoxine hydrochloride	400 "
Guanine	1.0 "	*p*-Aminobenzoic acid	20.0 "
Uracil	1.0 "	Biotin	0.5 "
Xanthine	1.0 "		
Inorganic salts A (see p. 1160)	1.0 ml.	pH adjusted to 6.6–6.8	

Equally satisfactory results may be obtained by measurement of the population of the microorganisms turbidimetrically after 18 hours of incubation at 33° C. A photoelectric colorimeter is employed for this purpose. The instrument is set at 100 per cent transmittancy with the solution from the assay tube containing no added pantothenic acid.

CALCULATION. Prepare a standard reference curve, employing the data obtained in the standard series by plotting on ordinary graph paper, μg. of calcium pantothenate as the abscissae and ml. of 0.1 N sodium hydroxide, or photometric density, as the ordinates. From this reference curve, estimate the "calcium pantothenate" content of each tube in the unknown series. Divide this value by the volume of the extract, originally placed in each tube, in order to obtain the concentration per ml. of extract. Determine the average of at least four values which differ from each other by no more than 10 per cent. Calculate the pantothenic acid content of sample using the formula:

$$C \times \frac{D}{G} \times 0.92 = \mu\text{g. pantothenic acid per g. of sample}$$

where C is the concentration of calcium pantothenate per ml. of extract, D is the final volume to which the extract was diluted, G is the weight of the sample taken for analysis, and 0.92 is the factor for converting calcium pantothenate to pantothenic acid.

Comment. The microbiological determination of pantothenic acid in these enzymic digests, employing *Lactobacillus arabinosus* 17-5, gives values which are in agreement with microbiological procedures employing other microorganisms as well as with the biological chick assay.

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[230] Hydrochloric acid-hydrolyzed, "Norit"-treated, vitamin-free casein is employed. A 10 per cent solution of casein hydrolyzate suitable for this medium may be obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

PTEROYLGLUTAMIC ACID (FOLIC ACID)

In early investigations, this vitamin was described under various names including vitamin M, factor U, yeast Norit eluate factor, vitamin B_c, and *L. casei* factor. The multiplicity of the nomenclature was occasioned by the fact that various conjugates of pteroylglutamic acid exist in natural materials. These conjugates vary in their physical and chemical properties, and in their biological potencies.

Physiological and Clinical Aspects of Pteroylglutamic Acid. This vitamin is a growth factor for various bacteria. Its deficiency in chicks leads to slow growth, poor feathering, and macrocytic anemia. In rats a deficiency of pteroylglutamic acid may be produced by adding sulfonamides to purified diets. Anemia, leukopenia and agranulocytosis are produced and may be cured by administering pteroylglutamic acid. Monkeys on a purified diet develop a syndrome characterized by leukopenia, anemia, necrosis of the gums, loss of appetite, diarrhea, and eventual death. The syndrome responds either to pteroylglutamic acid or to pteroyltriglutamic acid or to citrovorum factor.

Pteroylglutamic acid produces a clinical response in the megaloblastic anemias. It is used in the treatment of sprue, nutritional megaloblastic anemia, and the megaloblastic anemias of pregnancy and infancy. The blood picture in these diseases is marked by a low erythrocyte count, reduced hemoglobin, high color and volume indices of the erythrocytes, and lowered leukocytic and platelet counts. Accompanying this is "megaloblastic arrest" in the bone marrow so that an increase of nucleated red cells, including more than 2 per cent megaloblasts, is seen in films prepared from fluid aspirated from sternal bone marrow. Pernicious anemia is included in the megaloblastic group, but the disease is accompanied by neurological changes which respond only to vitamin B_{12}. In sprue, the administration of pteroylglutamic acid either parentally or orally results first in the disappearance of the symptoms of glossitis. A reticulocyte peak is commonly reached in six to nine days. Increases occur in erythrocyte count, per cent hemoglobin, white cell count, and number of platelets in a manner similar to that described above for pernicious anemia. There is an improvement in the sense of well-being, an increase in appetite, a subsidence of diarrhea, and a gain in body weight. The bone marrow picture shows a disappearance of the more primitive red blood cells and a return of the differential white cell count to normal proportions.

Nutritional anemia and the macrocytic anemias of pregnancy and infancy show hemopoietic responses similar to those described for pernicious anemia and sprue.

Pteroylglutamic acid appears to be specific in the treatment of the megaloblastic anemia of pregnancy. Typical findings were described in a case by Day and co-workers,[231] and are illustrated in Fig. 280. The patient, a parturient woman, had a hemoglobin of 6.5 g. per cent and a red cell count of 1.7 million. The administration of liver extract and vitamin B_{12} did not relieve the anemia and appeared to aggravate the associated symptoms which included glossitis and diarrhea. The changes following administration of pteroylglutamic acid were accompanied by hunger, recession of edema, and disappearance of the other signs and symptoms, including return of the hemopoietic system to normal. The increased dietary need for pteroylglutamic acid in pregnancy is also manifested in "lactation leukopenia"[232] in rats. The rats show blood changes due to a deficiency of pteroylglutamic acid in diets which suffice for maintenance but do not meet the extra demand occasioned by lactation.

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[231] Day, Hall, and Pease: *Proc. Staff Meetings Mayo Clinic*, **24**, 119 (1949).

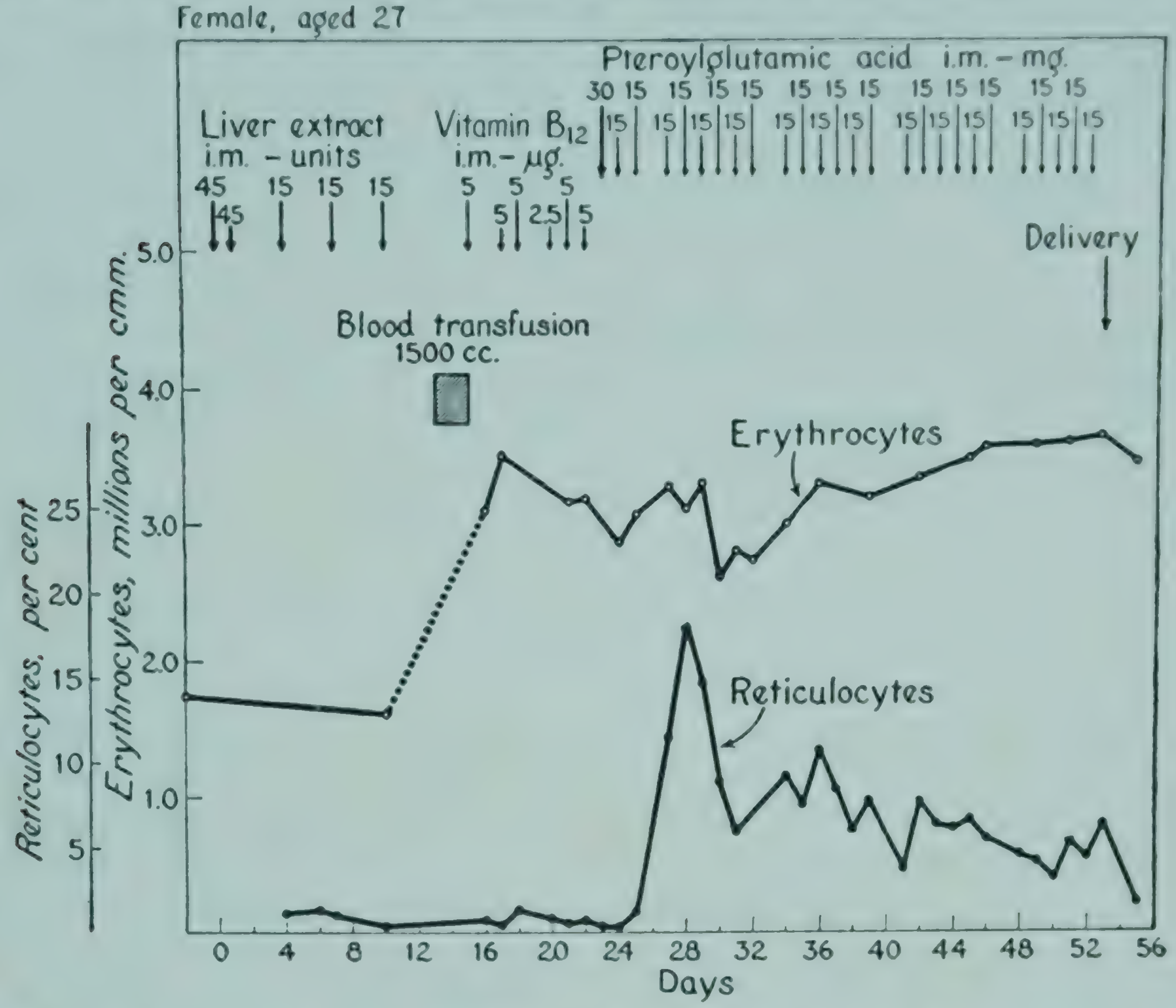

FIG. 280. ABSENCE OF HEMATOPOIETIC RESPONSE TO LIVER EXTRACT AND TO VITAMIN B_{12}, FOLLOWED BY PROMPT RESPONSE TO PTEROYLGLUTAMIC ACID. Courtesy, Drs. Day, Hall, and Pease.

Relation of Pteroylglutamic Acid to Vitamin B_{12}. These two antianemic substances show some unique clinical interrelationships. The blood and bone marrow changes in pernicious anemia respond to either pteroylglutamic acid or vitamin B_{12} even though no dietary deficiencies of pteroylglutamic acid may exist. The uptake of pteroylglutamic acid from the digestive tract of patients with pernicious anemia appears to be normal, but these patients cannot utilize vitamin B_{12} adequately owing to a functional deficiency of "intrinsic factor." The megaloblastic anemia of infancy responds uniformly to pteroylglutamic acid but does not respond consistently to vitamin B_{12}. These clinical phenomena find some interesting analogies in biochemistry; for example, both pteroylglutamic acid and vitamin B_{12} are concerned in biological reactions involving the formation of methionine from homocystine.[233]

Distribution of Pteroylglutamic Acid. Pteroylglutamic acid and its close relative, the citrovorum factor, are widely distributed in biological materials, often as polyglutamic acid conjugates. Good dietary sources include green leaves, asparagus, liver, yeast, cowpeas, and soybeans. The distribution of "folic acid activity" in foods has been exhaustively studied by microbiological and chick assays.[234] The name "folic acid" was originally defined in terms of a factor in spinach showing biological activity for *Streptococcus fecalis*. This factor was probably a mixture of pteroylglutamic acid and "citrovorum factor" which are interchangeable with respect to their biological activity for *S. fecalis* and are both present in spinach and many other natural materials.

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[232] Nelson and Evans: *J. Nutrition*, **38**, 11 (1949).

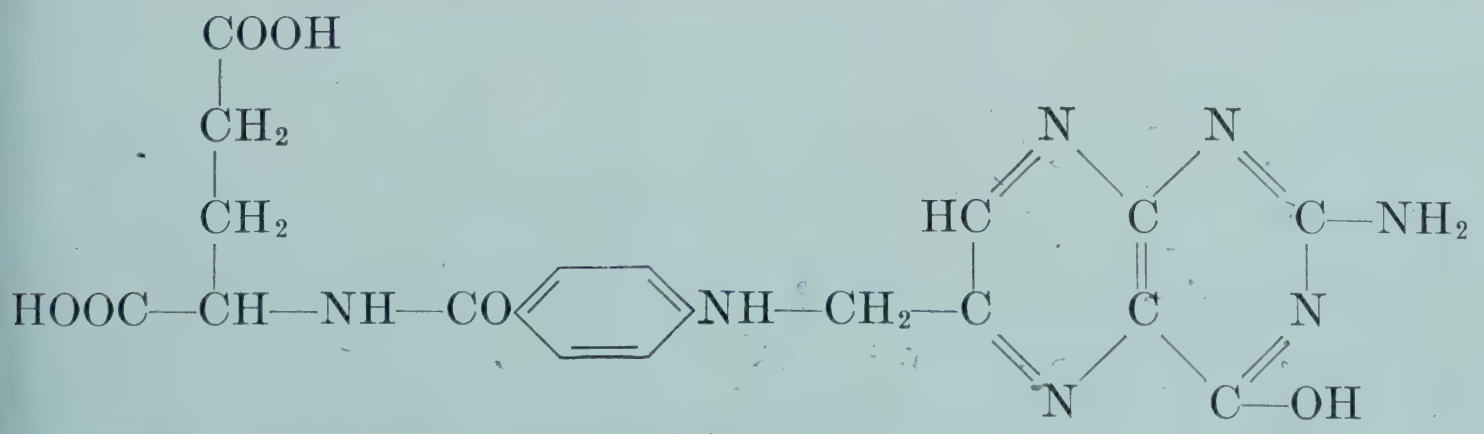
[233] Bennett: *Science*, **110**, 589 (1949).

THE FOLIC ACID ACTIVITY OF FOODS (UNDRIED)
EXPRESSED IN TERMS OF PTEROYLGLUTAMIC ACID*

Food	*Mg. per Kg.*	*Food*	*Mg. per Kg.*
Apples	0.01	Greens, various types	0.2 –1.1
Asparagus	0.9 –1.4	Lamb, leg	0.03
Barley	0.40	Lamb liver	2.8
Beans, dried	1–2	Lettuce head	0.03–0.11
" green	0.1 –0.4	Milk	0.06
" lima	0.1 –0.6	Oats	0.24
Beef kidney	0.6	Oranges	0.05
" liver	2.8 –3.1	Peas	0.1 –0.4
" round	0.06–0.17	Pork liver	2.2
Brewer's dried yeast	20	" loin	0.03
Broccoli	0.2 –1.5	Potatoes	0.02–0.19
Cabbage	0.1 –0.7	Raisins	0.01
Cheese, cheddar	0.2 –0.3	Rice, brown	0.1 –0.3
" cottage	0.2 –0.3	Soybeans	1.4 –2.7
Chicken liver	3.8	Squash	0.02–0.24
" meat	0.03	Strawberries	0.05
Corn	0.23	Sweet potatoes	0.05–0.19
Cowpeas	3.3 –4.8	Tomatoes	0.01–0.16
Egg white	0.004	Turkey meat	0.1
" yolk	0.13	Turnips	0.04
		Wheat	0.3 –0.4

* Condensed from *Agricultural Handbook No. 29*, U.S. Dept. of Agriculture.

Chemistry of Pteroylglutamic Acid. The structure and synthesis of pteroylglutamic acid were described in 1946.[235] The molecule consists of a pteridyl group connected in methylene linkage through *p*-aminobenzoic acid to L(+)glutamic acid and the structural formula is as follows:



Pteroylglutamic acid

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[234] *Folic Acid Content of Foods* (*Agricultural Handbook No. 29*), Washington, D. C., U.S. Government Printing Office, September 1951.

[235] Angier, *et al.*, *Science*, **102**, 227 (1945); **103**, 667 (1946).

The pteridyl group is chemically related to xanthopterin, a yellow pigment which is present in certain natural materials including urine and the wings of butterflies.

Pteroylglutamic acid is a substance of low toxicity as measured by toxicological tests with mice, rats, guinea pigs, rabbits, cats, and dogs. The LD_{50} for mice and rats is about 0.5 g. per kg. body weight when injected intravenously.

A second form of vitamin, "fermentation *L. casei* factor," isolated from an aerobic fermentation of an organism of the genus *Corynebacterium*, has been identified as pteroyldi-γ-glutamylglutamic acid. Upon partial degradation with alkali, it yields DL-pteroylglutamic acid. "Fermentation *L. casei* factor" is relatively inactive as a growth factor for *S. fecalis* R by comparison with pteroylglutamic acid. However, on a molar basis, it appears to be just as active as pteroylglutamic acid for *L. casei*, for chicks, for rats, and for monkeys. When the *p*-aminobenzoyl-glutamic-acid radical in the pteroylglutamic acid molecule is replaced by *p*-aminobenzoic acid, the resultant substance is termed "pteroic acid." This substance is active for *S. fecalis* R but not for *L. casei* or for animals.

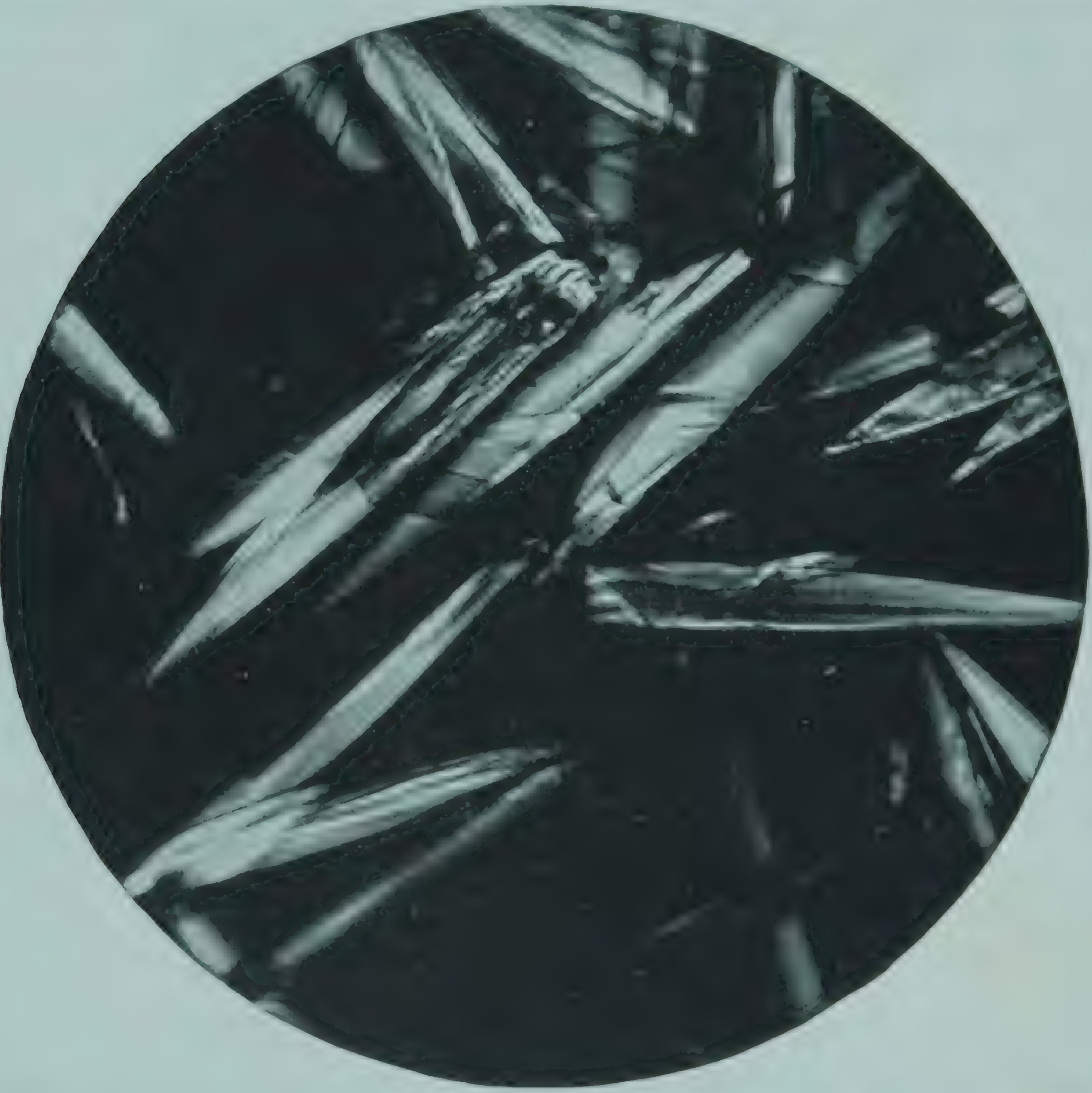

FIG. 281. SYNTHETIC PTEROYLGLUTAMIC ACID CRYSTALS, VIEWED THROUGH CROSSED POLARIZERS.

Photographed by Dr. A. F. Kirkpatrick, Stamford Laboratories, American Cyanamid Co. Courtesy, Dr. T. H. Jukes.

Other members of the pteroylglutamic acid family of vitamins are pteroylhexa-γ-glutamylglutamic acid (previously known as the vitamin B_c conjugate); N-formyl pteroic acid (rhizopterin or *S. lactis* R factor) which is inactive for animals; and folinic acid (citrovorum factor, or leucovorin), discussed in the next section. The differences in biological potency among the various natural forms of pteroylglutamic acid raises a question concerning the significance of microbiological assays in which the preliminary digestion procedures liberate maximum amounts of the parent compound.

Pteroylglutamic acid is a yellow, tasteless substance which is only slightly soluble in water; about 0.01 mg. per ml. dissolves at room temperature. It is destroyed rapidly by boiling with dilute hydrochloric acid. Its sodium salt is fairly soluble. Light has a destructive action on the solution. Pteroylglutamic acid has an absorption spectrum which shows characteristic maxima in the ultraviolet range.[236] The substance crystallizes from water in the form of thin lenticular crystals (Fig. 281) which exhibit birefringence and parallel extinction.

DETERMINATION OF PTEROYLGLUTAMIC ACID

Microbiological Assay.[237] The assay is carried out with either *Lactobacillus casei* (ATCC #7469) or *Streptococcus fecalis* (ATCC #8043).

PTEROYLGLUTAMIC ACID STANDARD SOLUTION.

(a) *Preparation.* Wash 20 mg. of pteroylglutamic acid with water into a 100-ml. volumetric flask. Add 2 ml. 0.1 N NaOH. Shake until material is in solution. Add 5 ml. of 1.0 M sodium phosphate buffer, pH 7.0, and 25 ml. absolute ethanol, and make up to 100 ml. with water.

BASAL MEDIUM FOR ASSAY OF PTEROYLGLUTAMIC ACID WITH *Lactobacillus casei*[238]

Constituent	*Amount per 500 ml. double-strength medium*	*Constituent*	*Amount per 500 ml. double-strength medium*
	g.		mg.
Acid-hydrolyzed casein	5.0	Adenine sulfate	5.0
Sodium acetate	20.0	Guanine hydrochloride	5.0
Glucose	20.0	Uracil	5.0
Asparagine	0.30	Xanthine	10.0
Tryptophan	0.10	Glutathione	2.5
Cysteine	0.25	Thiamine hydrochloride	0.2
Tween 80 (239)	0.05	Riboflavin	0.5
K_2HPO_4	0.50	Calcium pantothenate	0.4
KH_2PO_4	0.50	Nicotinic acid	0.4
$MgSO_4 \cdot 7H_2O$	0.20	Pyridoxine hydrochloride	2.0
NaCl	0.01		
$FeSO_4 \cdot 7H_2O$	0.01	*p*-Aminobenzoic acid	0.5
$MnSO_4 \cdot H_2O$	0.10	Biotin	0.01

Glucose, cysteine, and glutathione are added as solids. Other ingredients are added in the form of solutions. Combine ingredients, adjust to pH 6.8 and make up to 500 ml. with water.

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[236] Stokstad: *J. Biol. Chem.*, **149**, 573 (1943).

[237] Flynn, Williams, O'Dell, and Hogan: *Anal. Chem.*, **23**, 180 (1951).

[238] For assay with *Streptococcus fecalis*, omit sodium acetate and KH_2PO_4. Add 27.5 g. sodium citrate (dihydrate) and change amount of K_2HPO_4 to 3.10 g.

[239] Obtained from Atlas Powder Company, Wilmington, Del.

(b) *Dilutions.* Dilute to 0.001 μg. per ml. for assay with *L. casei;* to 0.005 μg. per ml. for assay with *S. fecalis.*

(c) *Assay Levels of Diluted Standard Solutions.* 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0, 5.0 ml. for *L. casei;* 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 3.0 ml. for *S. fecalis.*

The preparation of the various constituents of the medium is as follows:

Vitamin-free, Acid-hydrolyzed Casein.[240] Labco "vitamin-free" casein (300 g.) is refluxed at least 12 hours with 3 liters 20 per cent HCl. It is evaporated under vacuum to a thick syrup, then diluted to 3 liters with distilled water. This concentration procedure is repeated twice. The final concentrate is diluted to 1 liter. The pH is adjusted to 2.5 with KOH. The mixture is stirred with 30 g. activated carbon (Darco G 60) for 15 minutes, then filtered with the aid of "Filter-cel." The pH is adjusted to 6.8 with KOH after the volume of solution is brought to 3 liters, and the charcoal treatment and filtration are repeated. One ml. of the solution contains the equivalent of 100 mg. original casein.

Adenine and Guanine. These are dissolved together in a small amount of 1.0 N HCl with heat and diluted to 1 mg. per ml.

Xanthine and Uracil. These are dissolved in dilute NH_4OH and diluted to 1 mg. per ml.

Tryptophan. Dissolve in a small amount of 1.0 N HCl and dilute to 10 mg. per ml.

Asparagine. One g. asparagine is dissolved in 100 ml. water.

Vitamins. Four mg. thiamine hydrochloride, 10 mg. riboflavin, 8 mg. calcium pantothenate, 8 mg. nicotinic acid, 40 mg. pyridoxine hydrochloride, 10 mg. *p*-aminobenzoic acid, and 200 μg. biotin are dissolved in 100 ml. distilled water and stored in the cold room under toluene. One ml. of this solution is added per 100 ml. double-strength medium.

Preparation of sample solutions. Each sample for assay is blended with 150 ml. 0.05 M phosphate buffer pH 7.2 per g. of sample (dry basis). Caprylic alcohol is added to prevent foaming, and the mixture is autoclaved 15 minutes at 15 pounds. It is then cooled and incubated with desiccated chicken pancreas (Difco) using 20 mg. of dry pancreas per g. dry weight of sample under toluene for 24 hours at 37° C. After incubation the samples are autoclaved 5 minutes at 15 pounds, cooled and filtered. The samples together with the standard pteroylglutamic acid dilutions are pipetted into 18 × 150 mm. test tubes and brought to a volume of 5 ml. with water; 5 ml. of double-strength basal medium is added per tube; the tubes are capped with metal or glass caps and then are sterilized for 15 minutes at 15 pounds pressure. After cooling to room temperature the tubes are inoculated as described below.

Preparation of the Inoculum. The inoculum medium for *S. fecalis* contains in addition to the basal medium, 0.5 μg. of pteroylglutamic acid, 200 ml. tomato juice, 5 g. Bacto tryptone, 0.5 g. Wilson's liver fraction L[241] and water to 1,000 ml. The inoculum medium for *L. casei* contains in addition to the basal medium, 0.5 μg. pteroylglutamic acid, 0.5 g. Wilson's liver fraction L and water to 1,000 ml. The inoculum is incubated for 20–24 hours. The cells are centrifuged, the supernatant liquid is discarded, and the cells are resuspended in 0.9 per cent saline. One drop of this suspension is added to 10 ml. sterile 0.9 per cent saline and one drop of this cell suspension is added per tube. Microbiological assays with either organism may be incubated at 37° C. for the desired length of time. The growth response with *S. fecalis* may be determined after 16–20 hours' incubation by use of a photoelectric tur-

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[240] Kitay, McNutt, and Snell: *J. Bact.*, 59, 727 (1950).

[241] Other crude liver extracts may be used.

bidimeter or after 72 hours' incubation by titration of the acid formed with 0.1 N NaOH. The growth response with *L. casei* may be determined turbidimetrically after 40 hours' incubation, or after 72 hours' incubation by titration of the acid formed with 0.1 N NaOH. From these data a curve can be plotted relating growth to pteroylglutamic acid concentration, and from this response curve the potency of the unknown samples can be calculated.

CITROVORUM FACTOR (CF)

The occurrence of a growth factor for *Leuconostoc citrovorum* 8081 in certain natural materials including liver extract and yeast was reported in 1948.[242] The organism was shown to respond either to thymidine or to an unidentified "citrovorum factor" which was related to pteroylglutamic acid as indicated by the fact that a delayed and submaximal response was produced in the organism by massive doses of pteroylglutamic acid. It was shown that natural materials containing CF would reverse the inhibitory effect of 4-aminopteroylglutamic acid (aminopterin) for *Leuconostoc citrovorum* and that the administration of large doses of pteroylglutamic acid to animals produced many-fold increases in urinary CF. The factor was eventually synthesized by the formylation and reduction of pteroylglutamic acid.

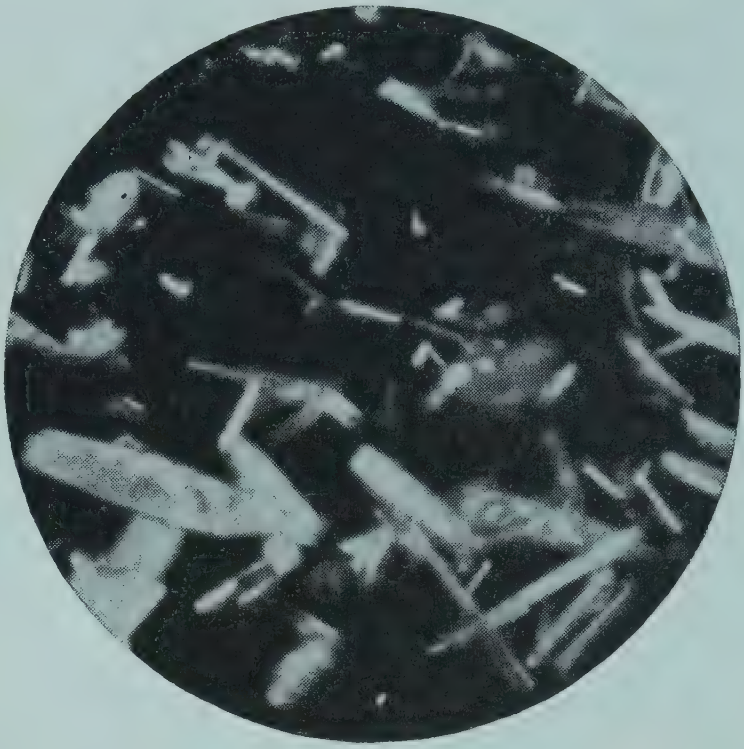

FIG. 282. CALCIUM LEUCOVORIN.
Courtesy, Dr. T. H. Jukes, American Cyanamid Co.

Chemistry of Citrovorum Factor (Leucovorin, Folinic Acid). It was shown that 5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid had the biological properties of the naturally occurring CF. The compound was synthesized from pteroylglutamic acid by hydrogenation in formic acid, alkaline treatment, and isolation of the active material.[243] A photomicrograph of crystals of the calcium salt of leucovorin is shown in Fig. 282.

Leucovorin produces responses in the megaloblastic anemias similar to those obtained with pteroylglutamic acid. It blocks the toxic effects of 4-aminopteroylglutamic acid and other "folic acid antagonists."

DETERMINATION OF CITROVORUM FACTOR

Microbiological Assay. The microbiological assay for the citrovorum factor is carried out with *Leuconostoc citrovorum* (ATCC #8081) following the procedures proposed by Sauberlich.[244]

CITROVORUM FACTOR (LEUCOVORIN, FOLINIC ACID) STANDARD SOLUTION.

(a) *Preparation.* A water solution containing 100 μg. DL-5-formyl-5, 6, 7, 8-tetrahydropteroylglutamic acid per ml. is prepared. An aliquot of this standard solution is diluted in stages to 1 millimicrogram per ml.

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[242] Sauberlich and Baumann: *J. Biol. Chem.*, **176**, 165 (1948).

[243] Flynn, *et al.: J. Am. Chem. Soc.*, **73**, 1979 (1951); Roth, *et al.: J. Am. Chem. Soc.*, **74**, 3247 (1952).

[244] Sauberlich and Baumann: *J. Biol. Chem.*, **176**, 165 (1948); Sauberlich: *J. Biol. Chem.*, **181**, 467 (1949).

(b) *Assay Levels of Diluted Standard Solutions.* 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml.

BASAL MEDIUM FOR ASSAY OF CITROVORUM FACTOR WITH *Leuconostoc citrovorum*

Constituent	*Amount per 500 ml. double-strength medium*	*Constituent*	*Amount per 500 ml. double-strength medium*
	g.		mg.
Acid-hydrolyzed casein	10.0	Adenine sulfate	10.0
Sodium acetate	20.0	Guanine hydrochloride	10.0
Glucose	25.0	Uracil	10.0
Asparagine	0.10	Xanthine	10.0
Tryptophan	0.10	Thiamine hydrochloride	0.5
Cystine	0.10	Riboflavin	0.5
NH_4Cl	3.0	Calcium pantothenate	0.5
K_2HPO_4	0.6	Nicotinic acid	1.0
KH_2PO_4	0.6	Pyridoxine hydrochloride	1.0
$MgSO_4 \cdot 7H_2O$	0.2	Pyridoxamine hydrochloride	0.3
NaCl	0.01	Pyridoxal	0.3
$FeSO_4 \cdot 7H_2O$	0.01	Biotin	0.001
$MnSO_4 \cdot H_2O$	0.01		

Combine ingredients, adjust to pH 6.8 and make up to 500 ml. with water.

The preparation of the various constituents of the medium is as follows:

VITAMIN-FREE, ACID-HYDROLYZED CASEIN.[245] Labco "vitamin-free" casein (300 g.) is refluxed at least 12 hours with 3 liters 20 per cent HCl. It is evaporated under vacuum to a thick syrup, then diluted to 3 liters with distilled water. This concentration procedure is repeated twice. The final concentrate is diluted to 1 liter. The pH is adjusted to 2.5 with KOH. The mixture is stirred with 30 g. of activated carbon (Darco G 60) for 15 minutes, then filtered with the aid of "Filter-cel." The pH is adjusted to 6.8 with KOH after the volume of solution is brought to 3 liters, and the charcoal treatment and filtration are repeated. One ml. of the solution contains the equivalent of 100 mg. of original casein.

ADENINE AND GUANINE. These are dissolved together in a small amount of 1.0 N HCl with heat and diluted to 1 mg. per ml.

XANTHINE AND URACIL. These are dissolved in dilute NH_4OH and diluted to 1 mg. per ml.

TRYPTOPHAN. Dissolve in a small amount of 1.0 N HCl and dilute to 10 mg. per ml.

ASPARAGINE. One g. of asparagine is dissolved in 100 ml. of water.

VITAMINS. Ten mg. thiamine hydrochloride, 20 mg. pyridoxine hydrochloride, 6 mg. pyridoxamine hydrochloride, 6 mg. pyridoxal, 10 mg. calcium pantothenate, 10 mg. riboflavin, 20 mg. nicotinic acid, and 0.02 mg. biotin are dissolved in 100 ml. distilled water and stored in the cold room under toluene. One ml. of this solution is added per 100 ml. of double-strength medium.

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[245] Kitay, McNutt, and Snell: *J. Bact.*, **59**, 727 (1950).

Preparation of sample solutions. **Each sample for assay is blended with 150 ml. 0.05 M phosphate buffer (pH 7.2) per g. of sample (dry basis). Caprylic alcohol is added to prevent foaming, and the mixture is autoclaved 15 minutes at 15 pounds. It is then cooled and incubated with desiccated chicken pancreas (Difco) using 20 mg. dry pancreas per g. dry weight of sample under toluene for 24 hours at 37° C. After incubation the samples are autoclaved 5 minutes at 15 pounds, cooled, and filtered. The samples, together with the standard citrovorum factor dilutions, are pipetted into 12 × 100 mm. test tubes and brought to a volume of 1 ml. with water; 1 ml. double-strength basal medium is added per tube. The tubes are capped with metal or glass caps and then are sterilized for 15 minutes at 15 pounds pressure. After cooling to room temperature, the tubes are inoculated as described below.**

Preparation of the Inoculum. **An inoculum is prepared by growing the organism at 37° C. for 24 hours on the basal medium plus 10 mg. Wilson's liver fraction L[246] per tube. The cells are centrifuged, the supernatant liquid is discarded, and the cells are resuspended in 0.9 per cent saline. One drop of this suspension is added to 10 ml. sterile 0.9 per cent saline, and one drop of this cell suspension is added per tube. The assay tubes are incubated at 37° C. for 20–24 hours. The growth response is measured by recording the optical density of the cultures with the photoelectric turbidimeter. From these data a curve can be plotted relating growth to citrovorum factor, and from this response curve the potency of the unknown samples can be calculated.**

BIOTIN

A striking example of how the isolation of an active principle in pure form can lead to coordination of a vast amount of seemingly unrelated observations may be seen in the story of biotin. Kögl and Tönnis[247] in 1936 reported the isolation from egg yolk of a yeast-growth factor (bios II) in the form of its methyl ester. They assigned the name *biotin* to the free acid. Recognition by West and Wilson[248] of the probable identity of this factor with coenzyme R, a growth and respiratory stimulant for the legume nodule organism Rhizobium, was followed by the demonstration by György, Melville, Burk, and du Vigneaud[249] of the identity of biotin with both coenzyme R and vitamin H. The latter had been previously reported to protect against a form of dermatitis induced by feeding raw egg white. The identification of biotin also illustrates the contribution to the advance in nutritional science made possible by the application of microbiological methods. The structure of biotin was established by du Vigneaud and associates.

In terms of the minimum protective dosage, biotin is one of the most potent physiological substances known, less than 0.03 μg. per day being sufficient for the rat. The role of biotin in nutrition is not clearly understood, but various investigations suggest that it may function as a coenzyme in certain enzymatic reactions associated with decarboxylation and carbon dioxide fixation. The high concentration of biotin in embryonic and tumor tissue has attracted considerable interest in the possible clinical significance of this vitamin.

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[246] Other crude liver extracts may be used.
[247] Kögl and Tönnis: *Z. physiol. Chem.*, **242**, 43 (1936).
[248] West and Wilson: *Science*, **89**, 608 (1939).
[249] György, Melville, Burk, and du Vigneaud: *Science*, **91**, 243 (1940).

Physiological Properties of Biotin. Biotin has been shown to be an essential nutrient for various lower organisms (including yeast, molds, bacteria, and fungi), for the rat, chick, turkey, monkey, rabbit, dog, and guinea pig, and for man. Deficiencies of this vitamin are difficult to produce experimentally because it may be synthesized by intestinal flora in higher animals. Deficiency symptoms may be induced, however, by feeding materials which either combine with the biotin to form nonabsorbable complexes (such as avidin of egg white, see below) or by feeding sulfa drugs which interfere with bacterial synthesis of the vitamin in the intestines.

In 1927 Boas discovered that the inclusion of large amounts of raw (but not of cooked) egg white in the diet of rats resulted in loss of hair, loss of weight, dermatitis, and death. It was also found that certain foods could cure or prevent these symptoms. Raw egg white contains a distinctive protein, later designated as *avidin*, capable of combining stoichiometrically with biotin, thus preventing its absorption from the digestive tract or its utilization by yeast. Symptoms of a biotin deficiency may be induced even in man by feeding a sufficient amount of raw egg white. Crystallized avidin (mol. wt. 70,000) has 15,000 times the biotin-combining power of raw egg white. Though the biotin-avidin complex is not absorbed from the gastrointestinal tract, the compound is biologically active when administered parenterally.

Biotin deficiency in animals is associated with the development of dermatitis, loss of fur, disturbances of the nervous system, and death. Biotin appears to be required for normal gestation and lactation in the rat and mouse as well as for normal development of the chick embryo. A marked increase in the biotin content of the blood of sexually immature chicks was observed following the administration of estrogen; subsequent administration of progesterone caused no change in blood biotin but produced a rise in the avidin content of the oviduct. These observations suggest a role of avidin and biotin in reproduction. The "spectacled eye" condition in rats (due to loss of hair around the eyes) may be associated with biotin deficiency. In the chick the deficiency symptoms dermatitis and perosis are similar to those of pantothenic acid deficiency. That vitamin interrelationships are involved in intestinal synthesis is illustrated by the observation that a pantothenic acid deficiency produced in rats on a diet containing succinylsulfathiazole could be relieved by administering folic acid and biotin.[250]

That biotin may play a part in the synthesis of oleic acid is suggested by studies with certain lactobacilli.[251] For example, *L. arabinosus* can multiply in the absence of biotin provided oleate and aspartate are present. Aspartic acid has a pronounced sparing action on the requirement of microorganisms for biotin. If just sufficient biotin is present to permit synthesis of oleic acid, aspartic acid can be replaced by oxalacetic acid. The microbial growth-stimulating effect of oleic acid is also manifested by certain detergents, e.g., Tween 80 (the polyoxyethylene derivative of sorbitan oleate). The significance of these observations in mammalian nutrition has not been established.

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[250] Wright and Welch: *Science*, **97**, 426 (1943).

[251] Potter and Elvehjem: *J. Biol. Chem.*, **172**, 531 (1948); Williams and Fieger: *J. Biol. Chem.*, **170**, 399 (1947); Williams, Broquist, and Snell: *J. Biol. Chem.*, **170**, 619 (1947); Broquist and Snell: *J. Biol. Chem.*, **188**, 431 (1951).

The protective effect of liver or yeast concentrates against butter yellow (dimethylaminoazobenzene) hepatoma in rats has been found to be unrelated to their content of biotin. In fact pure biotin was reported to increase the incidence of tumors in susceptible mice.[252] Egg white affords some protection against tumors in rats, but this is independent of the biotin-avidin relationship.[253] Addition of biotin to a diet containing butter yellow, but otherwise protective against tumor development, accelerated carcinogenesis; however, when the diet was conducive to tumor development, this effect was not observed.[254]

Most microorganisms require biotin in the form of the free acid, though the methyl ester is biologically active for yeast and partially so for *Lactobacillus casei.* The diaminocarboxylic acid obtained from biotin by the hydrolysis of the urea portion of the molecule possesses 10 per cent of the activity of the original vitamin. Oxidation to the sulfoxide does not affect the activity of biotin, whereas conversion to the sulfone causes considerable inhibition of the growth stimulation of yeast. Parenteral administration of biotin is three to five times more effective than oral administration in the treatment of deficiency symptoms.

The presence in urine of a substance having biotin potency for yeast which is not inhibited by avidin has been reported. The existence of other similar substances has been demonstrated, but in no instance have they been shown to be chemically similar to or related to biotin. Interference by such substances in the microbiological assay for biotin may be avoided by utilizing the ability of avidin to combine specifically with biotin.

Storage and Synthesis of Biotin. Biotin may be stored in the liver and kidneys, although newborn infants have no such reserves. It is probable that infants obtain some of their biotin from bacterial synthesis in the gastrointestinal tract. That such synthesis furnishes a considerable proportion of the biotin requirements of higher organisms is indicated by the fact that humans have been known to excrete via stools and urine three to six times as much biotin as they ingest. Rats do not require an external supply of biotin and excrete more than they consume. The vitamin is synthesized to a considerable extent in the rumen of the calf and cow. In the case of the rat the oral administration of sulfaguanidine or succinylsulfathiazole produces symptoms of biotin deficiency which are curable by administration of biotin.

Distribution of Biotin.[255] Biotin is present in most animal tissues but in higher concentration in liver, kidneys, egg yolk, yeast, pancreas, and milk. It is also found in vegetables, grains, nuts, feeds, pollens, and molasses. Biotin occurs naturally in combined forms, among which is a complex isolated from yeast and designated *biocytin.*[256] It is ε-N-biotinyl-L-lysine. Biocytin has the activity of biotin for *L. casei* but not for *L. arabinosus.* Biotin exists in the free state in fruits and grasses, partly bound in nuts, vegetables, and grains, and mostly in combined form in yeast and liver. For assay with microorganisms it may be liberated by autolysis, acid hydrolysis, or enzymatic digestion.

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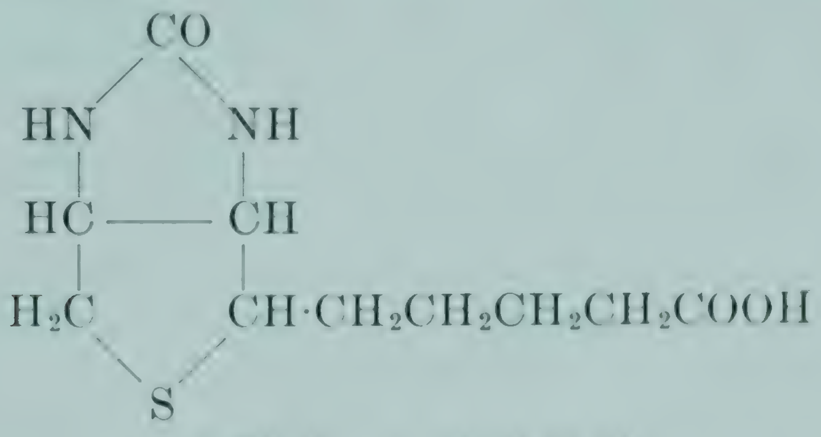
[252] du Vigneaud, *et al.: Science*, **95**, 174 (1942).

[253] Kline, Miller, and Rusch: *Cancer Res.*, **5**, 641 (1945).

[254] Harris, Krahl, and Clowes: *Cancer Res.*, **7**, 176 (1947).

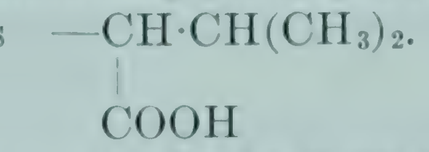
[255] For quantitative data see Lampen, Bahler, and Peterson: *J. Nutrition*. **23**, 11 (1943); cheese—Sullivan, Nielsen and Jarmol: *ibid.*, **25**, 463 (1943); meats—Schweigert, Nielsen, McIntire, and Elvehjem: *ibid.*, **26**, 65 (1943); milk—Lawrence, Herrington, and Maynard: *ibid.*, **32**, 73 (1946); Bowden and Peterson: *J. Biol. Chem.*, **178**, 533 (1949).

Chemistry of Biotin.



β-Biotin ($C_{10}H_{16}O_3N_2S$)

***cis*-Hexahydro-2-oxo-1-H-thieno(3,4)imidazole-4-valeric acid**

The structural formula shown is that of β-biotin, isolated from liver or milk. α-Biotin, from egg yolk, differs only in respect to the side chain which, instead of *n*-valeric acid, is  $\underset{\displaystyle \text{COOH}}{\text{—CH}}\cdot\text{CH}(\text{CH}_3)_2$.

The biotins are crystalline compounds soluble in water and alcohol and insoluble in chloroform, ether, and petroleum ether. The α- and β-biotins melt at 220° and 231° C., respectively. They have specific rotations $[\alpha]_D^{25°}$ of +51° and +91°, respectively, and an isoelectric point at pH 3–3.5. Biotin has an ultraviolet absorption maximum at 234 mμ with a specific extinction coefficient of 42.5. It has a molecular weight of 244.3. The stability of biotin toward heat in the presence of acid or alkali has not yet been established. It has been reported that biotin in natural materials resists autoclaving with strong mineral acids (4–6 N HCl for two hours at 120° C.), a procedure used for the liberation of the vitamin in natural materials for microbiological assay. Some authors, however, have reported a loss of biotin by this hydrolytic procedure. In natural materials the vitamin is destroyed by heating with strong alkali, and is even less stable to this treatment in pure solutions. Both free and combined biotin are inactivated by oxidizing agents. Though esterification impairs the biological activity of biotin for microorganisms, acylation or alkylation do not affect it. Synthetic β-biotin is produced commercially.

In oxybiotin the sulfur atom is replaced by oxygen. Oxybiotin possesses biological properties similar to biotin but in considerably less degree. It has about one-fourth the growth-promoting activity of biotin for *L. helveticus* and *S. cerevisiae* and half the activity for *L. arabinosus;* it is one-third as active in curing biotin deficiency in chicks and even less effective against egg-white injury in the rat.

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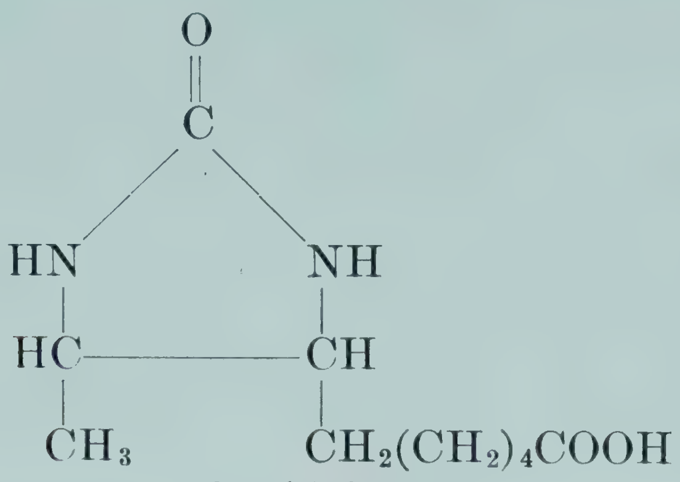
[256] Wright, *et al.: J. Am. Chem. Soc.,* **72,** 1048 (1950).

Dethiobiotin, the natural precursor of biotin, is believed to be derived in part from pimelic acid ($COOH \cdot (CH_2)_5 \cdot COOH$). It can be made by reducing biotin with Raney nickel, a reaction which leaves the furan ring intact.



Dethiobiotin

Dethiobiotin is as active as biotin for yeast, *E. coli*, and various molds, but not for several species of lactobacilli, and has very little of the activity of biotin against egg-white injury in the rat.

DETERMINATION OF BIOTIN

Biotin may be determined microbiologically by measurement of its effect upon the growth of microorganisms. The method of Snell, Eakin, and Williams[257] involves measurement of the growth stimulation of *Saccharomyces cerevisiae*. The procedure is similar to that described on p. 1185. The extremely high physiological potency of biotin is illustrated by the fact that it stimulates yeast growth when present in a concentration as low as one part in 5×10^{11} parts of medium. Biotin may also be determined by its growth stimulation of other microorganisms including *Lactobacillus arabinosus* and *Lactobacillus casei*. A rat assay such as was used in studies of the anti-egg-white-injury factor (vitamin H) may also be employed, though it requires large groups of animals and is not as satisfactory as the microbiological determination. One mg. of biotin methyl ester is equivalent to 27,000 units of vitamin H, the unit being the minimum daily dose required to cure the egg-white dermatosis in rats in 30 days. One great difficulty in the rat assay is that of producing biotin deficiencies, since rats obtain a considerable portion of their biotin from products of bacterial synthesis in their intestines. The assay for biotin using chicks is somewhat more satisfactory because they require greater amounts from dietary sources so that the deficiency is easily produced. Of the various assay methods, the microbiological procedure is generally preferred. See p. 1131.

VITAMIN B_{12} (COBALAMIN)

Vitamin B_{12}, the anti-pernicious-anemia factor of concentrated liver extracts and the major vitamin in sources of the "animal protein factor," was isolated in 1948 and found to be a cobalt coordination compound or "chelate." A second form, which was isolated in 1949, proved to be biologically interchangeable with the first. Later, these forms were identified as cyano (B_{12}) and hydroxo ($B_{12a} = B_{12b}$[258]) forms of the organometallic molecule containing cobalt. The known role of cobalt in the nutrition of ruminants could now be explained by the presence of this element in the molecule of these "cobalamins" which are synthesized by microorganisms in the rumen. The identity of cobalamin with the "extrinsic factor" of pernicious anemia was demonstrated, and the production of cobalamin by industrial fermentation made a new source of the substance available in medicine and animal nutrition.

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[257] Snell, Eakin, and Williams: *Univ. Texas Pub. No. 4137*, 18 (1941).

The term "vitamin B_{12}" refers chemically to *cyanocobalamin* but is commonly used to designate the cobalamin group with respect to their biological effect. For example, the vitamin B_{12} content of liver extract is commonly expressed in terms of a measurement of cyanocobalamin plus hydroxocobalamin.

Physiological and Clinical Aspects of Cobalamin. This vitamin is a growth factor for certain lactic acid bacteria and for the algal flagellate *Euglena gracilis*. The deficiency in young rats and chicks is seen when diets low in the vitamin are fed during the maternal period and is accentuated by raising the level of protein in such diets or by the addition of thyroid hormone to the diets fed to young animals. The deficiency in young rats is marked by a high mortality which is most prominent during the suckling period and is associated with leukopenia and uremia. The hatchability of the eggs of hens on deficient diets falls to low levels, and there is slow growth and a high mortality in newly hatched chicks.

Pernicious anemia is the best-known example of the result of vitamin B_{12} deficiency. In this disease, which is peculiar to the human species, the patient by some degenerative process is deprived of an "intrinsic factor" which is present in normal gastric juice. This "intrinsic factor" is needed for the uptake of vitamin B_{12} ("extrinsic factor") from the digestive tract. The disease is marked by a macrocytic anemia, by leukopenia, by megaloblastic changes in the bone marrow, and usually by subacute combined degeneration of the spinal cord. The signs and symptoms are reversed by administration of cobalamin. As little as 1 or 2 μg. daily will produce a therapeutic response when injected, as will 5 μg. daily by mouth when given with intrinsic factor or a single dose of 3000 μg. orally without intrinsic factor. Pteroylglutamic acid will restore the blood and bone marrow pictures to normal in doses of a few milligrams daily when given either orally or by injection but this treatment fails to stem the progress of, or to alleviate, the neural changes which respond only to cobalamin. If, however, these changes are not of recent origin, they are refractory to even large doses of cobalamin.

Vitamin B_{12} deficiency in pigs is marked by slow growth and is accompanied by nervousness and irritability, but not by macrocytic anemia. A wasting disease in ruminants known to be endemic in many areas was identified in 1935 as being due to a deficiency of inorganic cobalt in the soil, with a consequent lack of this element in the forage. The disease in sheep has been described as accompanied by listlessness, anemia, loss of appetite, and weakness progressing to a fatal termination. The disease can be prevented or arrested by cobalt salts when they are administered orally, but not when injected. Vitamin B_{12} is effective when either fed or injected, and it is thought that cobalt deficiency in the diet of ruminants leads to a failure in the fermentation process by which vitamin B_{12} is normally produced by the rumen microflora.

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[258] These designations were assigned by different groups of investigators before the identity of the compound was established.

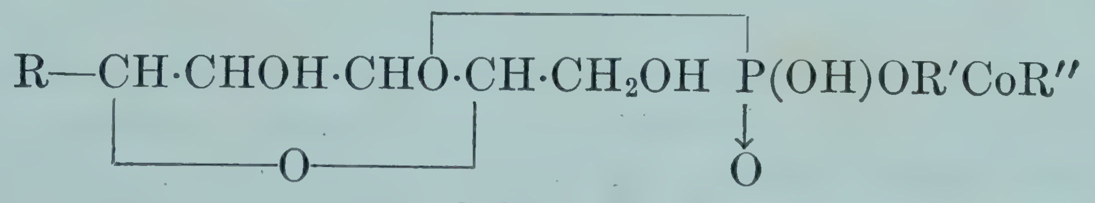
A source of the "labile methyl" group such as choline, betaine, or dimethylthetin will enable rats to grow on a "labile-methyl-free" purified diet, containing homocystine and succinylsulfathiazole, without adding vitamin B_{12} or pteroylglutamic acid. However, the rats will grow at a rate of 0.8 to 1.0 g. daily without a dietary source of "labile methyl" if both vitamin B_{12} and pteroylglutamic acid are added to the diet.[259] This relates these two substances to biological reactions involving precursors such as serine and glycine as sources of "formate," supplying a single-carbon fragment for the methylation of homocystine to form methionine. It was found by Shive[260] that the inhibitory effect of sulfanilamide on the growth of *E. coli* was overcome either by vitamin B_{12} at a level of 0.3 μg. per liter of culture medium or by methionine at a level of 90 mg., but not by homocystine. A function of vitamin B_{12} in the formation of methionine from homocystine was thus demonstrated.

Vitamin B_{12} and pteroylglutamic acid both appear to "spare" the requirement of the chick for choline and to improve the utilization of homocystine by the chick on methionine-deficient diets.

Vitamin B_{12} appears to catalyze the formation of the purine and pyrimidine deoxyribosides which are present in deoxyribonucleic acid. These deoxyribosides can replace vitamin B_{12} in the growth of certain bacteria including *L. leichmannii*.

The vitamin is formed by certain bacteria in the digestive tract and by soil and marine microorganisms. Its formation in the rumen doubtless accounts for its presence in beef liver. Vitamin B_{12} is characteristically present in the internal organs and muscular tissues of animals and absent from the higher plants. The more important dietary sources of the vitamin are liver, kidney, lean meat, fish, clams, oysters, milk, and egg yolk.

Chemistry of the Cobalamins. The molecule consists of a large, highly stable cobalt complex (R′Co) esterified through phosphoric acid to a ribofuranoside group which in turn is linked through N to R, which is 5,6-dimethylbenzimidazole (as in vitamins B_{12}, B_{12b}, and B_{12c}) or adenine (as in "pseudo vitamin B_{12}"). An anion R″, which may be CN



$$\mathrm{R{-}CH\cdot CHOH\cdot CHO\cdot CH\cdot CH_2OH\quad \underset{\overset{\downarrow}{O}}{P}(OH)OR'CoR''}$$

Cobalamin

(in B_{12}), OH (in B_{12b}), NO (in B_{12c}), etc., and which is apparently not related to the biological activity of the substance, is attached by coordinate linkage to the cobalt atom. Prolonged acid hydrolysis splits the phosphate linkages and also liberates two D-1-amino-2-propanol groups from R′. Catalytic hydrogenation of vitamin B_{12} leads to the production of a brownish pigment which has an absorption band at 315 mμ.[261] Upon prolonged exposure to air, the brown pigment becomes converted to hydroxocobalamin (vitamin B_{12b}) which in turn may be changed to cyanocobalamin (vitamin B_{12}) by treatment with cyanide.

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[259] Bennett: *Science*, **110**, 589 (1949).

[260] Shive: *Ann. N. Y. Acad. Sci.*, **52**, 1212 (1950).

The composition of vitamin B_{12} is approximately $C_{61-64}H_{86-92}N_{14}O_{13}PCo$. It forms dark-red, needle-shaped crystals which in solution have absorption spectrum maxima at 278, 361, and 550 mμ. The crystals of vitamin B_{12b} are almost black, and absorption spectrum maxima occur at 274, 351, and 525 mμ. Both substances are readily soluble in water and insoluble in ether. They may be crystallized from acetone. They are readily destroyed by alkali. Dilute acid converts B_{12} to B_{12b}, and strong acid causes hydrolytic decomposition as described above. The cobalamins are readily adsorbed on charcoal, from which they may be eluted with appropriate solvents, and they may be purified by chromatography on silicic acid.

An analog, "pseudovitamin B_{12}," was produced by anaerobic fermentation of an organism isolated from bovine rumen contents.[262] Acid hydrolysis of this analog yielded adenine in place of the 5,6-dimethylbenzimidazole which is obtained from vitamin B_{12}; the other hydrolytic products could not be distinguished from those yielded by vitamin B_{12}. Pseudovitamin B_{12} was active for *L. leichmannii*, *E. coli*, and *Euglena gracilis*, but was inactive in pernicious anemia and for chicks and rats. Other microbiologically active derivatives of cobalamin, inactive for animals, have been detected in the feces of animals.[263] These substances may be breakdown products of vitamin B_{12}.

Determination of Vitamin B_{12}. This is usually carried out by biological assay. Among the organisms used are rats, chicks, *Euglena gracilis*, *L. leichmannii*, and *E. coli* mutant No. 113-3. A correction for purine and pyrimidine deoxyribosides must be made if *L. leichmannii* is used. Chemical procedures have been described based on the addition of cyanide to cobalamin and the subsequent photolytic liberation of the cyanide, followed by its collection and determination.

Method of U.S. Pharmacopeia XIV—Modified.[263a] In the determination of vitamin B_{12} more than usual care must be taken, owing to the minute amount of the vitamin used in the assay tubes. All glassware must be carefully washed, then rinsed ten times or more with distilled water. To remove last traces of contaminants from glassware it is well to heat it in an oven at 200° C. for an hour.

TEST SOLUTION OF MATERIAL TO BE ASSAYED. For liquid preparations or pharmaceutical products, place a sample of suitable size, accurately measured, in a 500-ml. flask, add water to make 400 ml., adjust pH to 6.0, and make to volume of 500 ml. For food or feed products, heat in an autoclave for 15 minutes at 121° C., one g. or ml. of sample in 25 ml. of a freshly prepared bisulfite solution (13.6 g. monobasic potassium phosphate and 10 g. sodium metabisulfite, dissolved in water and made to 1 liter). After cooling, dilute an aliquot of the clear supernatant with water so that the final test solution contains vitamin B_{12} activity approximately equivalent to 0.02 millimicrogram of cyanocobalamin. The bisulfite aids in stabilizing naturally occurring analogs of the vitamin. The amount of bisulfite in the assay tube should not exceed 0.10 mg. (or 0.02 mg. per ml.).

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[261] Diehl and Murie: *Iowa State Coll. J. Sci.*, **26**, 555 (1952).

[262] Dion, Calkius, and Pfiffner: *J. Am. Chem. Soc.*, **74**, 1108 (1952).

[263] Wijmenga: Thesis, University of Utrecht, 1951; Ford, *et al.*: *Nature*, **171**, 150 (1953).

[263a] Grateful acknowledgment for permission to use this method is made to Dr. Lloyd C. Miller and the Board of Trustees of the U.S. Pharmacopeia, Inc.

STANDARD CYANOCOBALAMIN SOLUTION. Prepare a stock solution by dissolving in 25 per cent alcohol an accurately weighed quantity of U.S.P. Cyanocobalamin Reference Standard, so that each ml. contains 1.0 millimicrogram of cyanocobalamin. On the day it is to be used, dilute 10 ml. of the Stock Solution to 500 ml. with water. Each ml. contains 0.02 millimicrogram of cyanocobalamin.

BASAL MEDIUM STOCK SOLUTION

Acid-hydrolyzed Casein Solution	25 ml.
Cystine-Tryptophan Solution	25 ml.
Asparagine Solution	5 ml.
Adenine-Guanine-Uracil Solution	5 ml.
Xanthine Solution	5 ml.
Riboflavin-Thiamine-Biotin–Nicotinic Acid Solution	10 ml.
p-Aminobenzoic Acid–Calcium Pantothenate–Pyridoxine-Pyridoxal-Pyridoxamine–Folic Acid Solution	10 ml.
Salt Solution A	5 ml.
Salt Solution B	5 ml.
Polysorbate 80 Solution	5 ml.
Dextrose, Anhydrous	10 g.
Sodium Acetate, Anhydrous	5 g.
Ascorbic Acid	1 g.

Dissolve the dextrose, sodium acetate, and ascorbic acid in the solutions, previously mixed; add about 50 ml. of water, adjust to a pH of 6.0 with sodium hydroxide solution, and finally add water to make 250 ml.

ACID-HYDROLYZED CASEIN SOLUTION. Mix 100 g. vitamin-free casein with 500 ml. dilute hydrochloric acid (1 in 2) and reflux the mixture for 8 to 12 hours. Remove the hydrochloric acid from the mixture by distillation under reduced pressure until a thick paste remains. Redissolve the resulting paste in water, adjust the solution to a pH of 3.5 (±0.1) with sodium hydroxide solution, and add water to make 1000 ml. Add 20 g. activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal, stir for 1 hour, and filter. Repeat the treatment with activated charcoal. Store under toluene in a refrigerator at a temperature not below 10° C. Filter the solution if a precipitate forms upon storage.

CYSTINE-TRYPTOPHAN SOLUTION. Dissolve 400 mg. each of L-cystine and D,L-tryptophan in 100 ml. N HCl.

ASPARAGINE SOLUTION. Dissolve 2.0 g. L-asparagine in water to make 200 ml. Store under toluene in a refrigerator.

ADENINE-GUANINE-URACIL SOLUTION. Dissolve 0.2 g. each adenine sulfate, guanine hydrochloride, and uracil, with the aid of heat, in 10 ml. 20 per cent hydrochloric acid, cool, and add water to make 200 ml. Store under toluene in a refrigerator.

XANTHINE SOLUTION. Suspend 0.2 g. xanthine in 30 to 40 ml. water, heat to about 70° C., add 6.0 ml. ammonia Test Solution, and stir until the solid is dissolved. Cool, and add water to make 200 ml. Store under toluene in a refrigerator.

RIBOFLAVIN-THIAMINE-BIOTIN–NICOTINIC ACID SOLUTION. Prepare a solution in 0.02 N acetic acid, each ml. to contain 25 μg. riboflavin, 25 μg. thiamine hydrochloride, 0.2 μg. biotin, and 50 μg. nicotinic acid. Store, protected from light, under toluene in a refrigerator.

p-AMINOBENZOIC ACID-CALCIUM PANTOTHENATE-PYRIDOXINE-PYRIDOXAL-PYRIDOXAMINE-FOLIC ACID SOLUTION. Prepare a solution in 25 per cent neutralized alcohol each ml. to contain 50 μg. *p*-aminobenzoic acid, 25 μg. calcium pantothenate, 100 μg. pyridoxine hydrochloride, 100 μg. pyridoxal hydrochloride, 20 μg. pyridoxamine dihydrochloride, and 5 μg. folic acid. Store in a refrigerator.

SALT SOLUTION A. Dissolve 10 g. monobasic potassium phosphate and 10 g. dibasic potassium phosphate in water to make 200 ml. Add 2 drops of hydrochloric acid, and store under toluene.

SALT SOLUTION B. Dissolve 4.0 g. magnesium sulfate, 0.2 g. sodium chloride, 0.2 g. ferrous sulfate, and 0.2 g. manganese sulfate in water to make 200 ml. Add 2 drops of hydrochloric acid, and store under toluene.

POLYSORBATE 80 SOLUTION. Dissolve 20 g. polysorbate 80 in sufficient alcohol to make 200 ml. Store in a refrigerator.

TOMATO JUICE PREPARATION. Centrifuge 1000 ml. commercially canned tomato juice. Suspend 5 to 10 g. analytical filter-aid in the supernatant liquid and filter under reduced pressure, through a layer of analytical filter-aid of sufficient thickness so that a clear, straw-colored filtrate is obtained. Store under toluene in a refrigerator.

CULTURE MEDIUM. Dissolve 0.75 g. water-soluble yeast extract, 0.75 g. peptone, 1 g. anhydrous dextrose, and 0.2 g. potassium biphosphate in 60 to 70 ml. water. Add 10 ml. tomato juice preparation and 1 ml. polysorbate 80 solution. Adjust the solution to pH 6.8 with sodium hydroxide solution, and add water to make 100 ml. Place10-ml. portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave for 15 minutes at 121° to 123° C. (exhaust-line temperature).

SUSPENSION MEDIUM. Dilute a measured volume of basal medium stock solution with an equal volume of water. Place 10-ml. portions of the diluted medium in test tubes. Sterilize and cool as directed above for the culture medium.

STOCK CULTURE OF THE *Lactobacillus leichmannii*. To 100 ml. culture medium add 1.0 to 1.5 g. agar, and heat the mixture, with stirring, on a steam bath, until the agar dissolves. Add approximately 10-ml. portions of the hot solution to test tubes, plug the tubes with cotton, sterilize for 15 minutes in an autoclave at 121° to 123° C. (exhaust-line temperature), and allow the tubes to cool in an upright position. Prepare a stab culture from the pure culture of *L. leichmannii*, obtained from American Type Culture Collection[264] (No. 7830). Incubate 6 to 24 hours at any constant temperature between 30° and 37° C. Activate a newly obtained culture by making several daily transfers before use in an assay. Maintain the culture in an active state by transfer at least three times weekly.

INOCULUM. Make a transfer of cells from the stock culture to a sterile tube of culture medium, and incubate 6 to 24 hours at a constant temperature between 30° and 37° C. Under aseptic conditions centrifuge the culture, decant the supernatant, and suspend the cells in 10 ml. sterile suspension medium.

Procedure. **Cleanse hard glass test tubes, about 20 × 150 mm. in size, and other necessary glassware meticulously by suitable means because of the high sensitivity of the test organism to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents.**

To test tubes add in triplicate, 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 ml., respectively, of the Standard Cyanocobalamin Solution. To each tube add 5.0 ml. of basal medium stock solution and sufficient water to make 10 ml.

To similar test tubes add, in triplicate, respectively 1.0, 2.0, 3.0, and 4.0 ml.

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[264] American Type Culture Collection, 2029 M Street, N.W., Washington 6, D.C.

of the test solution of the material to be assayed. To each tube add 5.0 ml. of basal medium stock solution and sufficient water to make 10 ml.

After mixing, cover the tubes suitably to prevent bacterial contamination, and sterilize the tubes and contents in an autoclave for 5 minutes at 121° to 123° C. (exhaust-line temperature) arranging to reach this temperature in not more than 10 minutes. Cool as rapidly as practicable to avoid color formation resulting from overheating the medium. Take precautions to maintain uniformity of sterilizing conditions throughout the assay.

Aseptically inoculate each tube (except three tubes containing no Standard Cyanocobalamin Solution-uninoculated blank) with 1 drop of inoculum. Incubate for 6 to 24 hours, until maximum turbidity is obtained, as demonstrated by a lack of significant change during a two-hour period in the tubes containing the highest level of Standard Cyanocobalamin Solution (0.1 millimicrogram). Maximum turbidity is ordinarily obtained in 14 to 24 hours. Read the turbidity of the tubes in a suitable instrument, at a specific wavelength that is optimal for the instrument used. This will lie between 540 and 660 mμ. In taking the instrument reading, thoroughly mix the contents of each tube and transfer to optical glassware. Agitate each tube or cuvette to obtain a uniform suspension. A few seconds after agitation a steady state is reached in which the galvanometer needle remains constant for 30 seconds or more, allowing sufficient time for an instrument reading. A little practice will establish the proper time interval. With the inoculated blank in the instrument, set the meter to read 100 per cent transmittancy, read the transmittancy of the inoculated blank (the inoculated tubes to which no Standard Cyanocobalamin solution has been added). Disregard the results of an assay if contamination with a foreign organism is evident, or if the inoculated blank tubes give a reading of less than 90 per cent transmittancy (Evelyn or Lumetron), 80 per cent (Coleman), or 65 per cent (Beckman), thereby indicating interference due to vitamin B_{12} activity in the basal medium stock solution or inoculum. Then with the inoculated blank in the instrument set the meter to read 100 per cent transmittancy. Read the transmittancy of the tubes of the standard and sample series. Disregard the results of an assay if the transmittancy of the tubes containing the highest level of Standard Cyanocobalamin Solution (0.1 millimicrogram) is more than 65 per cent (Evelyn or Lumetron) or 50 per cent (Beckman or Coleman).

CALCULATION. Prepare a standard concentration response curve by plotting the per cent transmittancy readings for each level of the Standard Cyanocobalamin Solution used, against millimicrograms of cyanocobalamin contained in the respective tubes. Draw the smooth curve which by visual inspection appears to fit best the plotted points.

From this standard curve, determine by interpolation for each tube the amount of cyanocobalamin equivalent to the vitamin B_{12} activity of each ml. of the test solution of the material to be assayed.

Since in microbial assays occasional inexplicable aberrant values are obtained in individual tubes, inspect the series of values and set aside any which vary markedly from most of the series. Strike a provisional average of the remaining values, and set aside any of the latter which are less than 90 per cent and more than 110 per cent of the provisional average. If less than 10 of the 15 original values remain, the data are insufficient for calculating the potency; if 10 or more values remain, calculate the potency from the average of them. If the calculated potency of an assay is less than 75 per cent or more than 125 per cent of the assumed potency, the result is out of the critical range; for greater accuracy a reassay at a level more closely approximating the true value is necessary.

Determination of Cobalamin (Pad-Plate Method of Williams and Coworkers):[265] ***Principle.*** Cobalamin is determined by measurement of growth of *E. coli* mutant. The diameter of the growth zones on agar plate is measured and evaluated on a standard curve.

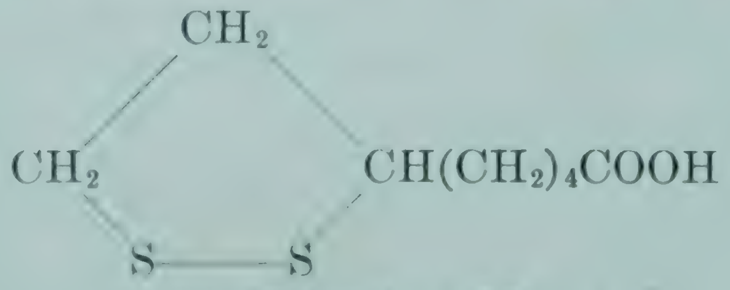
Distribution of Cobalamins. Good food sources include liver, kidney, lean meat, fish, milk, and oysters. The higher plants contain little or no vitamin B_{12}, but it is produced by many microorganisms, including molds of the *Streptomyces* group which are used for its industrial production. Ruminating animals appear to obtain the vitamin from fermentations which are carried out by the microflora of their digestive tract; this accounts for the presence of cobalamins in beef liver and also for the fact that in ruminants a lack of cobalt leads to a wasting disease caused by a deficiency of vitamin B_{12}.

THIOCTIC ACID (PROTOGEN, LIPOIC ACID)

This coenzymatic factor has not been shown to be essential in the diet of animals and should not, strictly speaking, be classified as a vitamin. However, thioctic acid has biochemical functions which relate it closely to the B vitamins.

The protozoon *Tetrahymena geleii* was shown by Dewey and Kidder[266] to need an unidentified factor present in liver and other natural materials. This factor was shown to contain a component which differed from the known vitamins and was named "protogen." Two chemically distinct forms were shown to exist.[267] In other investigations extracts prepared from yeast were found to be interchangeable with acetate in promoting the growth of *L. casei*[268] and to be needed for the oxidation of pyruvate by resting cells of a strain of *S. fecalis.*[269]

The crystallization and study of one form of the acetate-replacing factor, which was renamed "α-lipoic acid," showed the presence of sulfur[270] and a similar compound obtained from liver was also found[271] to contain sulfur. The synthesis of DL-6,8-dithiooctanoic acid (abbreviated to "6-thioctic acid") was accomplished following studies of the structure of the natural compound.[272] The biological activity of 6-thioctic



6-Thioctic Acid

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[265] Williams, Esposito, and Pierce: *Fed. Proc.*, **11**, 458 (1952).

[266] Dewey: *Proc. Soc. Exptl. Biol. Med.*, **46**, 482 (1941); *Biol. Bull.*, **87**, 107 (1944). Kidder and Dewey: *Biol. Bull.*, **87**, 121 (1944).

[267] Stokstad, Hoffman, Regan, Fordham, and Jukes: *Arch. Biochem.*, **20**, 75 (1949).

[268] Guirard, Snell, and Williams: *Arch. Biochem.*, **9**, 381 (1946).

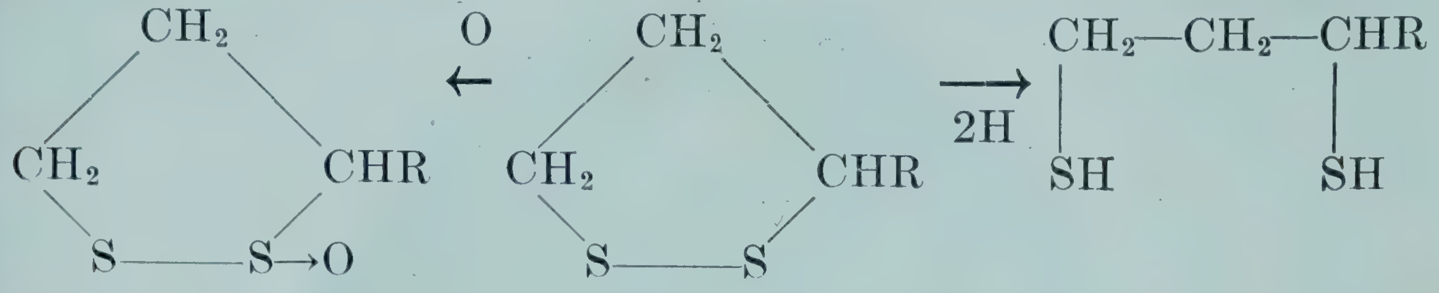
[269] O'Kane and Gunsalus: *J. Bact.*, **54**, 20 (1947).

[270] Reed, *et al.*: *Science*, **114**, 93 (1951); *J. Biol. Chem.*, **192**, 851, 859 (1951).

[271] Patterson, *et al.*: *J. Am. Chem. Soc.*, **73**, 5919 (1951).

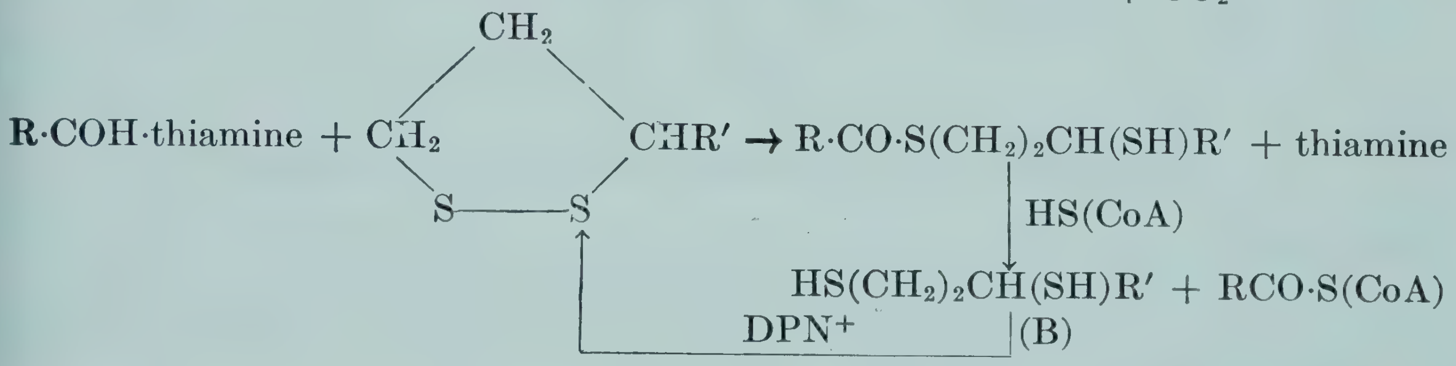
[272] Bullock, Brockman, Patterson, Pierce, and Stokstad: *J. Am. Chem. Soc.*, **74**, 3455 (1952).

acid is high; 0.4 parts per billion of purified culture medium is sufficient to produce half-maximum growth of *T. geleii*. It was suggested that the compound may be present in biological systems in acid amide linkage with thiamine pyrophosphate.[273] Oxidation and reduction of 6-thioctic acid give rise respectively to an oxide (protogen B or β-lipoic acid) and a dithiol:



Physiological Aspects of Thioctic Acid. The interchangeability of protogen with acetate in the nutrition of *S. fecalis* and the role of protogen in the oxidation of pyruvate were early observations which indicated the coenzymatic functions of the substance and its probable relation to co-carboxylase (diphosphothiamine). Approximately 6 moles of protogen and 1 mole of diphosphothiamine were found to be present in α-keto-glutaric acid oxidase,[274] and the presence of protogen in pyruvic oxidase was also noted,[275] suggesting a possibility that steps of the following type might occur in the oxidation of keto acids:[276]

$$\text{R·CO·COOH} + \text{thiamine} \rightarrow \text{R·COH·thiamine} + \text{CO}_2$$


R·COH·thiamine + CH₂
CH₂
CHR′ → R·CO·S(CH₂)₂CH(SH)R′ + thiamine
S—S
HS(CoA)
HS(CH₂)₂CH(SH)R′ + RCO·S(CoA)
DPN⁺
(B)

Oxidation of (B), the dihydrothioctic acid form of the coenzyme, restores the latter to its original condition.

Chemistry of Thioctic Acid. 6-Thioctic acid is a light-yellow crystalline solid, M.P. 60°–61° C. (Fig. 283). It is insoluble in water but readily dissolves in alcohol, acetone, ether, and many other organic solvents. The sodium salt is freely soluble in water. Thioctic acid is comparatively stable, but solutions tend to become oxidized by air to thiosulfinyloctanoic acid (oxythioctic acid, protogen B, or β-lipoic acid). The ultraviolet absorption spectrum shows a peak at 332 mμ (molar extinction coefficient ±160). Calvin and Massini[277] have suggested that light shifts the equilibrium of the thioctic-acid-containing coenzyme toward the reduced (dithiol) form, thus rendering it incapable of oxidatively decarboxylating pyruvic acid (newly formed from CO_2) to give rise to acetyl–coenzyme A required to bring this carbon into the tricarboxylic acid cycle. It was further suggested[278] that a biradical formed by dissociation of the disulfide bond in a strained five-membered disulfide-containing ring, such as in thioctic acid, is the form in which light energy travels from chlorophyll to the chemical reactions concerned with the fixation of carbon dioxide in photosynthesis (see also p. 58).

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[273] Reed and DeBusk: *J. Biol. Chem.*, **199**, 881 (1952).
[274] Sanadi, Littlefield, and Bock: *J. Biol. Chem.*, **197**, 851 (1952).
[275] Schweet and Cheslock: *J. Biol. Chem.*, **199**, 749 (1952).
[276] Reed and DeBusk: *J. Am. Chem. Soc.*, **75**, 1261 (1953).
[277] Calvin and Massini: *Experientia*, **8**, 445 (1952).

FIG. 283. THIOCTIC ACID.
Courtesy, Dr. T. H. Jukes, American Cyanamid Co.

DETERMINATION OF THIOCTIC ACID

Microbiological Assay. A number of microorganisms are now known which under appropriate conditions require thioctic acid for growth. *Corynebacterium bovis* (Strain B-187) has been shown by Stokstad *et al.*[279] to be suitable for the microbiological assay. The procedure is as follows:

THIOCTIC ACID STANDARD SOLUTION.

(a) *Preparation.* A water solution containing 1 μg. DL-6,8-dithiooctanoic acid per ml. is prepared. An aliquot of this standard solution is diluted to 1.0 millimicrogram per ml.

(b) *Assay Levels of Diluted Standard Solutions.* 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml.

The preparation of the various constituents of the medium is as follows:

VITAMIN-FREE, ACID-HYDROLYZED CASEIN.[280] Reflux 300 g. Labco "vitamin-free" casein at least 12 hours with 3 liters of 20 per cent HCl. Evaporate it under vacuum to a thick syrup, then dilute to 3 liters with distilled water. This concentration procedure is repeated twice. The final concentrate is diluted to 1 liter. The pH is adjusted to 2.5 with KOH. The mixture is stirred with 30 g. of activated carbon (Darco G 60) for 15 minutes, then filtered with the aid of "Filter-cel." The pH is adjusted to 6.8 with KOH after the volume of solution is brought to 3 liters, and the charcoal treatment and filtration are repeated. One ml. of the solution contains the equivalent of 100 mg. of original casein.

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[278] Calvin and Barltrop: *J. Am. Chem. Soc.*, **74**, 6153 (1952).
[279] Stokstad, *et al.*: *Proc. Soc. Exptl. Biol. Med.*, **74**, 571 (1950).
[280] Kitay, McNutt, and Snell: *J. Bact.*, **59**, 727 (1950).

ADENINE AND GUANINE. These are dissolved together in a small amount of 1.0 N HCl with heat and diluted to 1 mg. per ml.

URACIL. Dissolve in dilute NH_4OH and dilute to 1 mg. per ml.

TRYPTOPHAN. Dissolve in a small amount of 1.0 N HCl and dilute to 10 mg. per ml.

ASPARAGINE. Dissolve 1 g. asparagine in 100 ml. water.

VITAMINS. Dissolve 100 mg. *i*-inositol, 100 mg. choline chloride, 10 mg. calcium pantothenate, 10 mg. thiamine hydrochloride, 10 mg. nicotinamide, 10 mg. riboflavin, 10 mg. pyridoxine hydrochloride, 10 mg. *p*-aminobenzoic acid, 10 mg. pteroylglutamic acid, and 10 mg. biotin in 100 ml. distilled water, and store in the cold room under toluene. Add 1 ml. of this solution per 100 ml. of double-strength medium.

BASAL MEDIUM FOR ASSAY OF THIOCTIC ACID WITH *Corynebacterium bovis*

Constituent	*Amount per 500 Ml. Double-Strength Medium*	*Constituent*	*Amount per 500 Ml. Double-Strength Medium*
Acid-hydrolyzed casein	5 g.	*i*-Inositol	5 mg.
Glucose	5 g.	Choline chloride	5 "
Sodium acetate	2.5 g.	Calcium pantothenate	0.5 "
Speakman salt solution A	5 ml.	Thiamine	0.5 "
Speakman salt solution B	5 ml.	Nicotinamide	0.5 "
Asparagine	50 mg.	Riboflavin	0.5 "
DL-tryptophan	100 mg.	Pyridoxine	0.5 "
L-cystine	100 mg.	*p*-Aminobenzoic acid	0.5 "
Adenine sulfate	10 mg.	Pteroylglutamic acid	0.5 "
Guanine hydrochloride	10 mg.	Biotin	0.5 "
Uracil	10 mg.		

Combine ingredients, adjust to pH 6.8 and make up to 500 ml. with water.

Preparation of Sample Solutions. **If the thioctic acid in the test sample is in a "bound form," the sample should be autoclaved at 15 pounds pressure for 2 hours with 2 volumes of 8 N sulfuric acid, cooled, neutralized, and filtered to give a clear filtrate for assay. The assay samples, together with the standard dilutions of thioctic acid, are pipetted into 12 × 100 mm. test tubes and brought to a volume of 1 ml. with water; 1 ml. double-strength basal medium is added per tube, the tubes are capped with metal or glass caps, and then are sterilized for 15 minutes at 15 pounds pressure. After cooling to room temperature the tubes are inoculated as described below.**

Preparation of the Inoculum. **An inoculum is prepared by growing the organism at 25° C. for 48 hours on the basal medium plus 10 mg. Wilson's liver fraction L[281] per tube. The cells are centrifuged, resuspended in an equal volume of saline, and 1 drop is used per 2-ml. assay tube. The assay tubes are incubated at 25° C. for 72–88 hours. The growth response is measured by recording the optical density of the cultures with the photoelectric turbidimeter. From these data a curve can be plotted relating growth to thioctic acid concentration, and from this response curve the potency of the unknown samples can readily be calculated.**

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[281] Other crude liver extracts may be used.

Distribution of Thioctic Acid. The substance is widely distributed in biological materials. Liver, pancreas, yeast, green leaves, and soybean meal have been reported to be good sources. The thioctic acid content of the chick embryo increases rapidly during the final days of incubation, presumably indicating synthesis by the tissues.

PARA-AMINOBENZOIC ACID

The earliest indications of the nutritional importance of *p*-aminobenzoic acid were observations that the compound counteracted the bacteriostatic effect of sulfanilamide.[282] Its importance in the nutrition of microorganisms and of higher animals was established shortly thereafter, as well as its presence in certain natural materials, particularly yeast.[283]

Physiological and Clinical Aspects of *p*-Aminobenzoic Acid. *p*-Aminobenzoic acid is the "bridge" between pteroic and glutamic acids in the structure of folic acid. Although *p*-aminobenzoic acid (PABA) is frequently classed among the vitamins there is little to justify the belief that it plays a direct role in human nutrition. Evidence points rather to its essential role in microbial nutrition and hence to its stimulating effect on vitamin synthesis by intestinal microflora. Direct evidence of such bacterial synthesis is seen in the observation that excretion of *p*-aminobenzoic acid greatly exceeded dietary intake. (See p. 812.)

An excess of *p*-aminobenzoic acid inhibits the bacteriostatic effect of sulfonamides, possibly because of structural similarities (see Chapter 36). This antagonism obeys the mass action law, thus indicating that the inhibition is competitive. The antisulfonamide effect of the vitamin has been noted *in vitro*[284] as well as *in vivo* in mice infected with *Streptococcus hemolyticus*. Sulfonamide-resistant strains of *Staphylococcus* have been developed in which the resistance is proportional to the ability of the microorganism to synthesize *p*-aminobenzoic acid. *p*-Aminobenzoic acid interferes with the malaricidal action of sulfanilamide drugs, but not with that of quinine and atropine, which probably attack the microorganism through a different channel. The use of *p*-aminobenzoic acid derivatives as local anesthetics and their possible incompatibility with sulfonamides administered subsequently as bacteriostatic agents has been considered. Observations relating to the behavior of *p*-aminobenzoic acid under physiological conditions, however, indicate that *p*-aminobenzoic acid therapy probably does not interfere with subsequent administration of sulfonamides. Furthermore, it has been found that small amounts of *p*-aminobenzoic acid often potentiate sulfonamides.

p-Aminobenzoic acid is essential for normal growth[285] in the nonlactating rat,[286] for lactation in albino rats and in sows,[287] and for the maintenance of hair color in the black rat.[288] The injection of hydroquinone in cats or mice results in graying of the fur, which can be prevented or cured by *p*-aminobenzoic acid. The use of PABA in the treatment of nutritional achromotrichia in man, reported to be successful in a few isolated cases, has been refuted on the basis of more carefully controlled studies.[289] Anti-gray-hair properties have also been demonstrated for pantothenic acid, biotin, and folic acid. Stimulation of the growth of microorganisms by the administration of one of the vitamin B complex may cause an increased synthesis or utilization of another member by the intestinal flora. This makes it difficult to ascribe particular physiological effects to specific B vitamins when administered orally. Thus the action of *p*-aminobenzoic acid in the prevention of achromotrichia has been ascribed by some observers to alterations in the intestinal flora promoting the synthesis of pteroylglutamic acid or of other factors concerned with melanin formation.

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[282] Woods: *Brit. J. Exp. Path.*, **21**, 74 (1940); Rubbo and Gillespie: *Nature*, **146**, 838 (1940).

[283] Blanchard: *J. Biol. Chem.*, **140**, 919 (1941).

[284] Woods: *loc. cit.*

[285] Ansbacher: *Science*, **93**, 164 (1941).

[286] Unna, Richards, and Sampson: *J. Nutrition*, **22**, 553 (1941).

[287] Sure: *J. Nutrition*, **22**, 499 (1941).

In some species, *p*-aminobenzoic acid has been found to increase the physiological potency of insulin and penicillin. It may also play a role in the metabolism of hormones—for example, by inhibiting the production of thyroid hormones. In addition, the rate of enzymatic inactivation of stilbestrol by mushroom tyrosinase, as well as the oxidative destruction of epinephrine, are inhibited by *p*-aminobenzoic acid.

p-Aminobenzoic acid absorbs ultraviolet radiations (maximum 297.5 $m\mu$) in the range which produces sunburn and suntan in human skin.

p-Aminobenzoic acid participates in certain detoxication reactions. In rats PABA detoxifies high doses of pentavalent and trivalent arsenical drugs used in the treatment of various forms of syphilis, without interfering with their bacteriostatic potency. It has also been claimed to detoxify antimony compounds used in tropical diseases.

Large doses of *p*-aminobenzoic acid are toxic to dogs and mice. The oral administration of more than 1 g. per kg. body weight in dogs is fatal. On the other hand, the administration of 1.4 g. per kg. body weight to rats is nontoxic. Moderate doses are acetylated by man and excreted in the urine.

Distribution of *p*-Aminobenzoic Acid.[290] *p*-Aminobenzoic acid is present in most tissues. It occurs in nature in both the free and the combined form. Relatively high concentrations (several micrograms per gram) are found in yeast, liver, rice bran, rice polishings, and whole wheat. In the last it is present mostly in the germ. Milk contains about 0.1 mg. per liter.

Chemistry of *p*-Aminobenzoic Acid. *p*-Aminobenzoic acid was synthesized by Fischer[291] as early as 1863, by reduction of *p*-nitrobenzoic acid with ammonium sulfide. It crystallizes in colorless needles which melt at 186° C. It is soluble in water to the extent of 0.5 per cent at room temperature, but is freely soluble in alcohol or boiling water.

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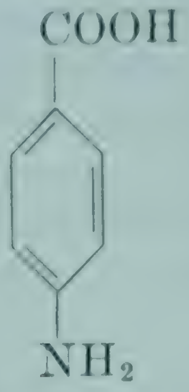
[288] Martin and Ansbacher: *J. Biol. Chem.*, **138,** 441 (1941).

[289] Brandalcone, Main, and Steele: *Proc. Soc. Exptl. Biol. Med.*, **53,** 47 (1943); Eller and Diaz: *N.Y. State J. Med.*, **43,** 1331 (1943).

[290] For quantitative data see Landy and Dicken: *J. Biol. Chem.*, **146,** 109 (1942); Mitchell, Isbell, and Thompson: *ibid.*, **147,** 485 (1943) and **148,** 281 (1943).

[291] Fischer: *Ann.*, **127,** 142 (1863).



p-Aminobenzoic acid

DETERMINATION OF *p*-AMINOBENZOIC ACID

No satisfactory chemical method is available for the determination of *p*-aminobenzoic acid. It reacts with *p*-dimethylaminobenzaldehyde in glacial acetic acid to produce a compound having a yellow color. However, this reaction, as well as other coupling reactions, is nonspecific and is given by isomers of *p*-aminobenzoic acid as well as by aniline and its derivatives, and by sulfonamides. In the analysis of biological materials, the bound vitamin must first be freed by strong acid or alkaline hydrolysis.

p-Aminobenzoic acid is required for the growth of several microorganisms, including *Brucella abortus*, *Streptococcus hemolyticus*, and *Clostridium acetobutylicum*. Microbiological methods for the determination of PABA are based upon the growth stimulation of *Acetobacter suboxydans*[292] and of a *p-aminobenzoicless* mutant of *Neurospora crassa*.[293] Determinations may also be made employing organisms which do not require an external supply of the vitamin by measuring the degree of inhibition of the bacteriostatic effect of sulfa drugs resulting from the addition of *p*-aminobenzoic acid.

CHOLINE

Choline has occupied a prominent place in biochemical literature because of its relationship both to the phospholipides and to its acetyl ester. Acetylcholine, first studied as a synthetic product, then found in plant and animal tissues, plays an important role in the humoral transmission of parasympathetic and other nerve impulses to effector organs. More recently, choline has achieved added significance because of its role in the process of transmethylation (see below and p. 1029).

Choline, probably of phospholipide origin, was isolated independently by numerous investigators, and has been variously designated sinkalin, bilineurine, fagin, amanitin, and neurine, the latter term being now reserved for the unsaturated base, trimethylvinylammonium hydroxide.

Because of its biochemical function and its distribution in foods, choline is usually considered along with the vitamins of the B group. However, it not only lacks the specificity characteristic of the vitamins but is actually a structural component of fat and nerve tissue. Moreover it is not known to participate as a cofactor in an enzyme system. Hence choline is not, strictly speaking, a vitamin. Part of the syndrome of choline deficiency in animals can be cured by the administration of other methyl group donors, (e.g. methionine, betaine) or of vitamin B_{12} which plays a role in methyl group transfer.

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[292] Cheldelin and Bennett: *J. Biol. Chem.*, 161, 751 (1945).

[293] Agarwala and Peterson: *Arch. Biochem.*, 27, 304 (1950).

Physiological Properties of Choline. Choline is essential for normal growth of the rat, chick, and dog, and for lactation in the rat. Dietary deficiency has been demonstrated to be responsible for paralysis in the hind legs of nursing rats, inhibition of egg production in hens, slipped tendon (perosis) in chicks and young turkeys, and fatty liver in rats, dogs, rabbits, and pigs. The last-mentioned symptom is not observed in guinea pigs, whose low requirement for choline is attributed to a lack of hepatic choline oxidase. Choline deficiency in young rats produces an acute hemorrhagic lesion of the kidneys. Even after short exposure to this condition and subsequent restoration to an adequate diet, such rats have been reported later in life to develop hypertension with cardiac enlargement and persistent renal damage.[294]

Choline performs several physiological functions. It enters into the molecular structure of phospholipides and acetylcholine, and supplies labile methyl groups for transmethylation reactions. Phospholipides are concerned with the mobilization of fat in the body. In the absence of choline, neutral fat, and to some extent cholesterol esters, accumulate in the liver. Choline is also lipotropic in that it prevents fatty livers in depancreatized dogs. However, this condition, when induced in rats by high-cholesterol feeding, does not respond to choline feeding.

Certain specific compounds with labile methyl groups, or otherwise related in structure, can replace choline in some of its biological functions. Methionine mobilizes liver fat in a manner similar to choline, and both the D- and L-forms are equally effective. The mechanism probably involves the transfer of labile methyl groups from methionine to ethanolamine with the formation of choline. In support of this hypothesis is the observation that choline containing deuterium may be isolated from the animal organism after feeding methionine whose labile methyl group contains deuterium. Betaine and other lipotropic factors may also supply methyl groups to ethanolamine. Conversely, methionine, an essential amino acid, can be replaced by homocystine when choline, betaine, or dimethylethylammonium chloride is fed. The methyl-diethyl and the triethyl homologs do not support growth, but are strong lipotropic agents, and prevent the occurrence of hemorrhagic kidneys. Arsenocholine and sulfocholine likewise have lipotropic action and prevent renal hemorrhage, but neither of these compounds can methylate homocysteine. There is no diminution of activity when phosphorus is substituted for the nitrogen of choline. The hydroxyl group must be free, however, since ethers are inactive. The methyl groups of creatine, S-methyl cysteine, or of the betaines from threonine, serine, or allothreonine, are not available for transmethylation.

Choline serves as a methylating agent in the physiological process: guanidoacetic acid → creatinine. Here, too, methionine and betaine can replace choline.

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[294] Hartcroft and Best: *Brit. Med. J.*, 1, 423 (1949).

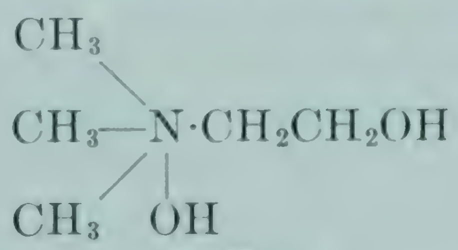
Choline is involved in the transmission of nerve impulses in the form of its acetyl ester, acetylcholine, a powerful agent which lowers the blood pressure. The organism maintains a delicate balance between acetylcholine and choline (the latter having only slight vasodilator effect) by means of specific enzyme systems which can acetylate to acetylcholine or hydrolyze to free choline when necessary.

Storage and Synthesis of Choline. Choline is present in the animal organism chiefly in the form of the phosphatides; i.e., the lecithins and sphingomyelins. The fatty matter of brain, kidney, and liver is distinguished from that of adipose tissue by the fact that it consists largely of phosphatides. Lecithin is the glyceryl ester of a pair of fatty acids and a substituted phosphoric acid group attached to a choline radical. Sphingomyelin contains a fatty acid, sphingosine, and a choline-phosphoric acid group. In beef liver, only 2 per cent of the total choline is present in the free form.

As discussed above, choline may be synthesized *in vivo* from ethanolamine and a methyl donor, methionine or betaine. Rats on a diet containing ample methionine, for example, need no dietary supply of choline.

Distribution of Choline.[295] The concentration of choline in anima tissues is proportional to their phospholipide constituents. Thus egg yolk, liver, kidneys, brain, heart, and nervous tissue are rich sources Good sources of choline are muscle tissue, green, leafy and leguminou vegetables, seed oil meals, and grain germs. In corn meal, 75 per cen of the choline is concentrated in the germ, while 50 per cent of the cholin in wheat is lost in the preparation of white flour. In general, seed meal and seeds are considerably better sources than the cereal grains. Butter, lard, and refined vegetable oils are almost devoid of choline. Mixed diet of man have been shown to provide 0.5 to 1.0 g. of choline per day, ar order of magnitude more nearly resembling that of individual amino acid than that of vitamins.

Chemistry of Choline. Choline was first isolated by Strecker in 184 and synthesized by Wurtz in 1867. It possesses the following structure



Choline

The free base is a colorless, crystalline, extremely hygroscopic com pound. It is a stronger base than ammonia and easily forms salts with acids. Pure choline, when heated, decomposes to trimethylamine an ethylene glycol. Aqueous solutions containing less than 4 per cent cholin may be heated without decomposition, but losses occur in more con centrated media. Choline is more stable to heat in acid than in neutra or alkaline solution. Choline dihydrogen citrate, choline chloride, an carbamyl choline chloride are used medicinally.

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[295] For quantitative data see Rhian, Evans, and St. John: *J. Nutrition*, **25**, 2 (1943 Engel: *ibid.*, **25**, 441 (1943); McIntire, Schweigert, and Elvehjem: *ibid.*, **28**, 219 (1944 Glick: *Cereal Chem.*, **22**, 95 (1945); Willstaedt, Borggard, and Lieck: *Z. Vitaminforsch.* 1 25 (1946); also reference footnote 296.

Choline is readily soluble in water, methyl alcohol, ethyl alcohol, and formaldehyde; slightly soluble in dry amyl alcohol, dry acetone, and chloroform; and insoluble in dry ether, petroleum ether, benzene, toluene, carbon bisulfide, and carbon tetrachloride. The salts are soluble in water and alcohol, forming aqueous solutions which are nearly neutral.

Choline may be precipitated from aqueous solution by potassium triiodide, phosphotungstic acid, phosphomolybdic acid, or reinecke salt. These precipitants, especially the last, have been employed in quantitative methods for its determination. Choline chloride also forms characteristic double salts with the chlorides of platinum, gold, and mercury.

DETERMINATION OF CHOLINE

Microbiological Assay* (*Method of Horowitz and Beadle*):[296] *Principle. Choline may be measured by the growth stimulation of a mutant strain of *Neurospora crassa* designated as *cholineless*, produced artificially by exposure to ultraviolet radiation. Methionine, which can replace choline for the microorganism, is removed by adsorption of the extract on "Permutit" followed by elution with sodium chloride solution. The microbiological procedure for the determination of choline is far more sensitive and possibly more specific than the chemical procedure.[297] The latter involves precipitation and isolation of the reineckate, followed by colorimetric measurement of the red pigment at 520 mμ in acetone solution.

***Procedure.* Heat 100 mg. of sample in an autoclave for 2 hours at 15 pounds pressure in 10 ml. 3 per cent sulfuric acid, or reflux for 7 hours. Neutralize to congo red with saturated barium hydroxide solution. Centrifuge and filter the supernatant through a Whatman No. 50 paper. Add 3 ml. distilled water to the precipitate and bring to a boil with stirring. Cool, centrifuge, and add the washing to the previous supernatant. Neutralize the solution to litmus with 1 N sodium hydroxide solution and dilute with distilled water to a concentration of approximately 15 μg. per ml. Pass 5 ml. of the neutralized extract[298] through a column 100 mm. long and 5 mm. wide (internal diameter) containing 1 g. of Permutit. Wash the column with 5 ml. of 0.3 per cent sodium chloride and discard the filtrate and washings. Elute the choline with 10 ml. of 5 per cent sodium chloride.[299]**

Prepare a basal medium having the following composition in g. per liter: ammonium tartrate 5, ammonium nitrate 1, monobasic potassium phosphate 1, magnesium sulfate·7H_2O 0.5, sodium chloride 0.1, calcium chloride 0.1, sucrose 20, biotin 5×10^{-6}. In addition, add the following trace elements as salts in mg. per liter: boron 0.01, molybdenum 0.02, iron 0.2, copper 0.1, manganese 0.02, and zinc 2.0.

Maintain stock cultures of the cholineless mutant No. 34486 of *Neurospora crassa* on agar slants composed of the basal medium plus 1.5 per cent agar, 0.2 per cent Difco yeast extract, 0.2 per cent malt extract, and 1 μg. per ml. of choline. For inoculum, prepare a spore suspension in a few ml. of distilled water.

Pipet 0.5, 1.0, and 2.0 ml. aliquots of the sodium chloride eluate in duplicate into 250-ml. Erlenmeyer flasks, and dilute each to 25 ml. with basal

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[296] Horowitz and Beadle: *J. Biol. Chem.*, **150**, 325 (1943).

[297] U.S. Pharmacopoeia XIII.

[298] If the solution is known to contain less than 3 μg. of choline per ml., pass 10 ml. through the column.

[299] If more filtrate is required in case the approximate choline content is unknown, adsorb two or more portions of the solution simultaneously on separate columns.

medium. The final concentration of choline should lie between 2.0 and 20 μg. per 25 ml. At the same time set up a series of standards having 0, 2, 4, 8, 12, 16, 20, 25, and 30 μg. of choline per flask. Autoclave the solutions at 15 pounds pressure for 10 minutes, cool, and add 1 drop of inoculum to each. Incubate at 25° C. for 3 days. At the end of this period, collect the mold pads on tared, fritted glass filters of medium porosity, and wash with distilled water. Dry in an oven at 90° C. and determine the dry weight.

CALCULATION. Prepare a reference curve from the data obtained with the standard series, plotting μg. of choline as the abscissa and weight of dry mold as the ordinate. From this curve read off the choline concentration, C, per ml. of eluate. Then

$$C \times \frac{10}{A} \times \frac{V}{0.1} = \mu\text{g. choline per g. of sample}$$

where V is the volume to which the original extract was diluted, and A is the volume of the aliquot which was passed through the "Permutit" column.

INOSITOL

Though inositol was long known to be a constituent of heart muscle, and of many plants (in the form of phytin, the calcium-magnesium salt of inositol hexaphosphate), its functional similarity to the vitamins was not recognized until recently. Eastcott[300] isolated from yeast the factor previously known as bios I and demonstrated its identity with inositol. The biologically active compound is optically inactive (designated *i*-inositol or *meso*-inositol) as distinguished from its biologically inert stereoisomers. In addition to its role as a yeast growth factor, inositol has been identified with the mouse alopecia factor of Woolley,[301] and the rat anti-spectacled-eye factor of Pavcek and Baum.[302]

Physiological Properties of Inositol. Though the metabolism or mode of action of inositol has not yet been elucidated, its importance in the nutrition of several species has been demonstrated. An external source is required by some yeasts and fungi, but not by all. Young mice on a deficient diet suffer an inhibition of growth and loss of hair, both of which are corrected by supplementation with inositol. Growth of a transplanted mouse tumor has been inhibited by inositol. It is also essential for proper growth in the chick and rat. The spectacled-eye condition in rats due to loss of hair about the eyes is a result of dietary deficiency. The vitamin also has lipotropic properties for this species, but more for the "cholesterol" than the "fat" type of fatty livers. Inositol stimulates gastrointestinal peristalsis in dogs. Though the vitamin must be present in the free state to be available to yeast, it is readily utilized by the mouse in natural bound forms such as phytin and soybean cephalin, as well as synthetic methyl inositol and inositol hexaacetate.

The metabolism of inositol is related to that of other vitamins. It has been suggested that pantothenic acid regulates absorption of inositol from the intestine, for a diet containing inositol produces symptoms of inositol deficiency if pantothenic acid is lacking. A relationship to *p*-aminobenzoic acid has also been established. In one series of experiments, black rats showed little signs of deficiency when both inositol and *p*-aminobenzoic acid were either absent or present, but exhibited typical symptoms when either nutrient alone was eliminated from the diet.

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[300] Eastcott: *J. Phys. Chem.*, **32**, 1094 (1928).

[301] Woolley: *J. Biol. Chem.*, **136**, 113 (1940).

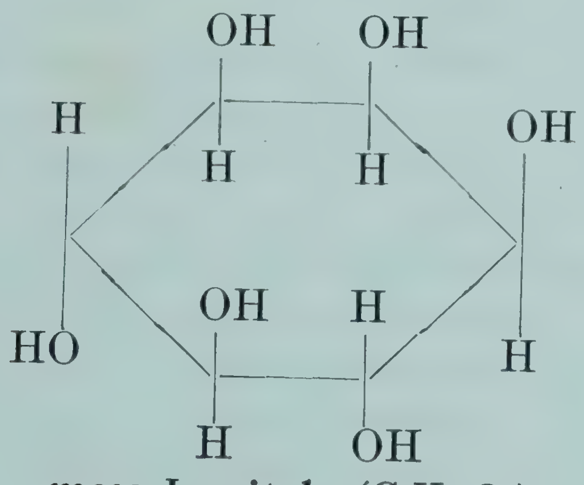
[302] Pavcek and Baum: *Science*, **92**, 384 (1940).

Because of its similarity in structure both to the carbohydrates and to the aromatics, inositol has been designated as a possible physiological link between these two classes of compounds.

Storage and Synthesis of Inositol. The wide distribution and variety of forms of inositol in animal and plant tissues indicate that it is present for some physiological purpose rather than merely for storage of phosphoric acid or hydroxyl groups. It has been reported that inositol may be synthesized by intestinal flora, a process stimulated by the presence of pantothenic acid.

Distribution of Inositol.[303] In animal tissues, high concentrations are found in kidney, heart, spleen, thyroid, and testes. Inositol is found in the free form in muscle and other tissues; hence the name muscle sugar. In liver and heart muscle it is found combined, probably with a protein. Excellent dietary sources are cereal brans and seeds (in the form of phytin) and fruits, particularly of the citrus variety. A compound of inositol has been found in the phosphatide fraction of the soybean.

Chemistry of Inositol. *meso*-Inositol is one of the eight *cis-trans* isomers of hexahydrocyclohexane, one of which occurs in optically active forms, making nine in all. It is a sweet-tasting crystalline compound and is isomeric in molecular formula with the hexose sugars. It crystallizes as the anhydride (M.P. 225°–226°) from water at temperatures above 50° C. and from anhydrous solvents. Below 50°, the dihydrate (M.P. 215°–216°) appears. The molecule is stable to strong acid and alkaline hydrolysis. It is very soluble in water but insoluble in absolute alcohol or ether. *meso*-Inositol is optically inactive.



***meso*-Inositol** **($C_6H_{12}O_6$)**

Determination of Inositol. The microbiological method for deternination of vitamin B_6, depending on the growth stimulation of *Saccharonyces cerevisiae* (p. 1185) or *carlsbergensis*[304] may be used for the deternination of inositol by simple modification of the basal medium to exlude inositol and include an excess of pyridoxine. High specificity is claimed for the microbiological assay using the *inositol-less* mutant of *Neurospora crassa* (No. 37401).[305]

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[303] For quantitative data see Woolley: *J. Biol. Chem.*, **140**, 453 (1941) and **147**, 581 (1943); ealock and Livermore: *J. Nutrition*, **25**, 265 (1943); Mollgaard, Lorenzen, Hansen, and hristensen: *Biochem. J.*, **40**, 589 (1946).

[304] Atkin, *et al.*: *Ind. Eng. Chem., Anal. Ed.*, **15**, 141 (1943).

ASCORBIC ACID (VITAMIN C)

Scurvy was once extremely common particularly among navigators on long voyages. Its etiological relationship to the absence of fresh fruits and vegetables was forecast as far back as the sixteenth century, when the curative value of a decoction of spruce needles, an herb called scurvy grass, and especially of "sower Oranges and Lemmons" were recognized. The regulation requiring British vessels to carry a supply of lemons or limes for their crews gave rise to the name Limehouse for London's shipping district, whence the term "limey" or "lime juicer" for a sailor.

The dietary origin of the disease was confirmed experimentally in guinea pigs by Holst and Frölich[306] in 1907, and the deficient factor was among the first to receive the designation of a vitamin. Outbreaks of scurvy among infants, prisoners, explorers, troops, and in war-ridden countries where the diets are restricted or unbalanced, continue to be reported. Frank scurvy is comparatively rare. Far more prevalent is the incidence of subclinical vitamin C deficiency manifested by vague and indefinite symptoms and diagnosed only with the aid of accurate dietary histories and laboratory determinations of the blood levels or urinary excretion of the vitamin. Several studies of the ascorbic acid nutrition of large population groups have revealed widespread subclinical vitamin C deficiency. In the examination of large segments of the population in Puerto Rico and Newfoundland, 58 and 72 per cent respectively of the individuals examined showed low plasma ascorbic acid levels.[307] The status of ascorbic acid nutrition shows seasonal variation, and depends upon the fluctuation of available dietary supplies. Because of the relatively high cost of fresh fruits and vegetables as compared with the less expensive cereal grains, subclinical vitamin C deficiency is more prevalent among low-income groups.

The isolation of crystalline vitamin C from lemon juice was first announced in 1932 by King and Waugh.[308] Szent-György[309] concluded from his own studies on adrenal cortex, cabbage, and citrus juices that vitamin C was identical with "hexuronic acid," but later, with the collaboration of Haworth, established a different structural configuration and named vitamin C "ascorbic acid" because of its antiscorbutic properties. Shortly after the structure of the vitamin was established, its synthesis was reported.[310] Ascorbic acid is an important article of commerce today not only for its nutrient and medicinal value but because it is a powerful antioxidant.

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[305] Beadle: *J. Biol. Chem.*, **156**, 683 (1944).

[306] Holst and Frölich: *J. Hyg.*, **7**, 634 (1907).

[307] Munsell, Cuadros, and Suarez: *J. Nutrition*, **28**, 383 (1944); McDevitt, Dove, Dove, and Wright: *Ann. Internal Med.*, **20**, 1 (1944).

[308] King and Waugh: *Science*, **75**, 357 (1932); *J. Biol. Chem.*, **97**, 325 (1932).

[309] Szent-György: *Biochem. J.*, **22**, 1387 (1928); Svirbely and Szent-György: *Nature* **129**, 576, 690 (1932); *Biochem. J.*, **26**, 865 (1932).

[310] Hirst, Percival, Smith, Haworth, *et al.*: *J. Chem. Soc.*, **10**, 1270 and 1419 (1933); Reichstein, Grüssner, and Oppenauer: *Helv. Chim. Acta*, **16**, 561 and 1019 (1933); Micheel and Kraft: *Z. physiol. Chem.*, **215**, 215 (1933).

Physiological and Clinical Aspects of Ascorbic Acid. Vitamin C is concerned fundamentally with the formation of intercellular substances, including the collagen of fibrous tissue structures, the matrices of bone, cartilage, and dentin, and all nonepithelial cement substance, including that of the vascular endothelium. In the absence of the protection afforded by this vitamin, the condition known as scurvy develops.

The onset of the disease is gradual in humans, a period of indolence, fleeting pains in the joints, and shortness of breath being followed by decline in weight and anemia. Soon the complexion becomes sallow, subcutaneous hemorrhages occur upon slight injury, the gums become spongy and bleed easily, the teeth become loose and fragile, and often there is marked edema of the extremities. Hemorrhage is a predominating feature of the disease, and when it occurs internally it is frequently the cause of death. Various methods have been devised to measure the reduction in capillary resistance which occurs (not exclusively, however) in scurvy. These tests[311] are based on the production of small, subcutaneous hemorrhages (*petechiae*) by applying suction or pressure to circumscribed skin areas.

Histological studies have emphasized the importance of the vitamin C content of the diet on tooth development.[312] Scurvy is attended by profound pathological changes in the teeth and gums, affecting particularly the vascular pulp and the periodontal and gingival tissues. Resorption of normal dentin and cementum occurs. In scurvy, bone formation stops, but resorption continues so that there is a resultant atrophy and bone fragility. Skeletal defects are demonstrated first at the costochondral junctions, where the lesions are similar to those of rickets.

The severity of the lesions observed in scurvy is dependent upon other factors, such as growth and mechanical stress. Gum damage occurs only when there are teeth, and hemorrhages appear chiefly from mechanical injuries or from the irritation of clothing. Bone lesions appear mainly at the joints and are particularly severe in infants and children. The response of scorbutic patients to treatment with ascorbic acid is dramatic. Histological evidence of bone repair may be observed within a few hours after administration of the vitamin, and signs of connective tissue regeneration in 24 hours. Evidence has been presented that an optimal level of ascorbic acid nutrition favors rapid repair of tissues in wounds and bone fractures, and is an important factor in determining resistance to infection. An outline of the clinical symptoms of ascorbic acid deficiency is given in the American Medical Association syllabus on p. 1292.

Ascorbic acid may play a role in endowing the organism with increased powers of resistance. This is suggested by studies with guinea pigs receiving injections of diphtheria toxin following graded allowances of ascorbic acid,[313] and by experiments in which sensitized guinea pigs fed liberal doses of ascorbic acid showed diminished skin hypersensitivity to arsphenamine.[314] The protective effect of ascorbic acid against arsenical toxicity has been reported to be greatly augmented by the simultaneous administration of the flavonoid derivative, hesperidin methyl chalcone.[315]

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[311] Göthlin: *Skand. Arch. Physiol.*, **61**, 225 (1931); Dalldorf: *Am. J. Diseases Child.*, **46**, 794 (1933); Cutter and Johnson: *J. Am. Med. Assoc.*, **105**, 505 (1935).

[312] Wolbach and Howe: *Arch. Path.*, **1**, 1 (1926); **5**, 239 (1928); Mellanby: *Physiol. Revs.*, **8**, 545 (1928); Hanke: *J. Nutrition*, **3**, 433 (1931); Fish and Harris: *Proc. Roy. Soc. London*, **223**, 489 (1933).

[313] King and Menten: *J. Nutrition*, **10**, 129 (1935); Greenwald and Harde: *Proc. Soc. Exptl. Biol. Med.*, **32**, 1157 (1935).

An increased requirement for vitamin C in man has been reported in Hodgkin's disease, protracted fevers, active rheumatic heart disease, and tuberculosis. In rats the synthesis and excretion of ascorbic acid is stimulated by feeding certain cyclic ketones related to the terpenes and certain simple aliphatic ketones.[316] This increased synthesis may be related to greater requirements for detoxification purposes. The beneficial effects of vitamin C in the detoxification of other unrelated poisons have been reported; e.g., lead and arsenic compounds, benzene, and the toxins of several pathogenic microorganisms.

Under normal conditions a dietary supply of ascorbic acid is required only by man, other primates, guinea pigs, and some microorganisms. However, the vitamin is essential for the normal development of most species, and is produced endogenously, thus resembling a hormone. Interference with the normal synthesis of the vitamin in the bovine species results in the impairment of the reproductive functions of both the male and female. Dietary deficiency of vitamin A results in decreased synthesis of ascorbic acid in the rat, though this relationship with vitamin A has not been observed in mature hens or bulls. In the latter species, the administration of chlorobutanol stimulates the synthesis of ascorbic acid, raises the plasma level, and restores fertility in the deficient animals.

The reduction of the blood level of ascorbic acid and the occurrence of a scurvylike syndrome in rats on a vitamin A- and C-free diet suggest a functional relationship between these vitamins.[317] Addition of ascorbic acid to the diet prevented or delayed the appearance of symptoms. Similar observations have been reported in foxes.[318] That ascorbic acid participates in the metabolism of other vitamins as well is indicated by its capacity for increasing the urinary excretion of citrovorum factor in rats and human subjects,[319] its influence on the storage of folic acid in the liver of chicks,[320] and its effect of relieving deficiency symptoms of rats on a ration lacking thiamine, riboflavin, or pantothenic acid.[321]

Synergism between ascorbic acid and vitamin A has been noted in the rat as well as between C and E in the guinea pig. Ascorbic acid enhances the growth-promoting effect of vitamin A in the rat in a manner similar to tocopherol. The mechanism may be through stabilization of vitamin A in the gastrointestinal tract. The administration of vitamin E increases the storage of dietary ascorbic acid in the organs of the guinea pig.

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[314] Sulzberger and Oser: *Proc. Soc. Exptl. Biol. Med.*, **32**, 716 (1934). See also Bundesen, *et al.*: *J. Am. Med. Assoc.*, **117**, 1692 (1941); McChesney, *et al.*: *J. Pharmacol. Exptl. Therap.*, **80**, 81 (1944).

[315] Friend and Ivy: *Proc. Soc. Exptl. Biol. Med.*, **67**, 374 (1948).

[316] Longenecker, Musulin, Tully, and King: *J. Biol. Chem.*, **129**, 453 (1939).

[317] Mayer and Krehl: *J. Nutrition*, **35**, 523 (1948); *Arch. Biochem.*, **16**, 313 (1948).

[318] Bassett, Loosli, and Wilke: *J. Nutrition*, **35**, 629 (1948).

[319] Welch, *et al.*: *J. Pharmacol. Exptl. Therap.*, **101**, 37 (1951); **103**, 403 (1951).

[320] Dietrich, *et al.*: *Proc. Soc. Exptl. Biol. Med.*, **75**, 130 (1950).

[321] Daft: *Fed. Proc.*, **10**, 380 (1951); McDaniel and Daft: *Fed. Proc.*, **10**, 387 (1951).

The ease with which ascorbic acid undergoes reversible oxidation-reduction has suggested that the vitamin plays a role in cellular respiration. The oxidation of ascorbic acid is catalyzed by ascorbic oxidase, found in plant juices. The reverse reaction, the reduction of dehydroascorbic acid to reduced ascorbic acid, is catalyzed by ascorbic reductase (an enzyme found in plant juices)[322] in the presence of reduced glutathione. Though no similar enzyme systems have been observed in animal tissues, the administration of dehydroascorbic acid to man is followed by increased urinary excretion of reduced vitamin C in an amount comparable to that which is excreted when the reduced form of the vitamin is fed. It is believed that this reduction occurs in the liver.

Certain *in vitro* findings lend credence to the view that ascorbic acid functions *in vivo* in oxidation-reduction reactions. Vitamin C plus minute amounts of iron cause a considerable increase in the oxygen uptake by phospholipides and by brain and liver suspensions. There is evidence, both *in vitro* and *in vivo*, that ascorbic acid functions as a coenzyme in the metabolism of tyrosine, although the exact nature of this action is not fully established.[323] A similar role is attributed to folic acid, the requirement for which appears to be increased in ascorbic acid deficient animals.

The action of urease, an enzyme which catalyzes the decomposition of urea to ammonia and carbon dioxide, is inhibited by vitamin C. This inhibition has been found to be due to the presence of small quantities of dehydroascorbic acid, and is prevented by cysteine, which reduces the dehydro form. Quinone, another oxidizing agent, can inhibit urease activity.

The recommended dietary allowances of ascorbic acid are shown on p. 1108. Approximately 1 mg. per day per kg. body weight is required for the maintenance of tissue saturation. These allowances are quite liberal, since clinical signs of scurvy do not appear even at considerably lower levels of intake. Considerable controversy exists as to the level of dietary intake of ascorbic acid necessary to insure adequate operation of the manifold functions in which this vitamin plays a role. Hence the recommendations of the National Research Council (p. 1108) provide for a substantial margin of safety. When healthy subjects have been subsisting on diets containing the recommended levels of intake for prolonged periods, the administration of a test dose of ascorbic acid (400 to 1000 mg.) is followed in the next 24 hours by an increase in the urinary excretion of from 25 to 50 per cent of the test dose. At lower levels of intake for prolonged periods there is a tendency for the body to conserve the test dose of the vitamin and lower excretion values are noted, indicating that body tissues are unsaturated.

No toxic symptoms are observed in man following the administration of large doses of ascorbic acid. One to 6 g. have been given orally and intravenously. Though ascorbic acid has been found in sweat, the amount lost through this channel even in excessive physical labor and in hot climates is not significant when the dietary intake approximates the recommended allowances.

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[322] Crook and Morgan: *Biochem. J.*, **38**, 10 (1944); Bukin: *Biochimia*, **8**, 60 (1943).

[323] Bessey, Lowe, and Salomon: *Ann. Rev. Biochem.*, **22**, 545 (1953).

Storage and Synthesis of Ascorbic Acid. As previously stated, all animal species other than man, monkeys, and guinea pigs, as well as higher plants and many microorganisms, are capable of synthesizing vitamin C. The promptness with which scurvy develops when the susceptible species are deprived of vitamin C would seem to indicate the absence of a large store in their bodies. In man, a diminished intake of ascorbic acid results in a prompt fall in the blood level of ascorbic acid, though tissue stores may not yet be depleted. Guinea pigs show histological symptoms of ascorbic acid deficiency after one week on a vitamin-C-free diet. Higher concentrations of the vitamin are found in tissues of high metabolic activity—e.g., the adrenal and pituitary glands and the intestinal wall. It is likely that the vitamin is present in these depots in order to meet tissue requirements, rather than for the purpose of storage. Certain animals which synthesize vitamin C store it in their livers in sufficient amounts to meet their immediate requirements. The fruits and vegetables consumed by man as sources of ascorbic acid represent storage depots for plants which synthesize the vitamin.

Distribution of Ascorbic Acid.[324] The outstanding sources of vitamin C are fresh fruits and vegetables. Special mention may be made of cabbage, cauliflower, kohlrabi, spinach, parsley, kale, broccoli, cresses, peppers, oranges, lemons, grapefruit, tangerines, limes, strawberries, and gooseberries. Other excellent dietary sources are cantaloupe, currants, papaya, persimmons, pineapple, asparagus, lima beans, green snap beans, and Brussels sprouts. Unfortunately, much of the vitamin C originally present in foods is lost through processing, so that for practical purposes the fruits and vegetables which are consumed raw contribute a major portion of the dietary ascorbic acid. For economic reasons, these are outside the reach of low-income groups which obtain their vitamin C from the relatively inexpensive potato. During the germination of legumes as well as of cereal grains, marked synthesis of vitamin C takes place from an unidentified precursor within the seed. Meat is low in vitamin C, although blood and glandular organs contain more than muscle. Milk varies in its content according to the cow's ration and hence the season of the year. Fresh raw milk is a good source of vitamin C, but most of it is lost in pasteurization, evaporation, or dehydration. Where milk is a major constituent of the diet, as it is for children, it is best to provide antiscorbutic protection from other sources; e.g., orange juice or synthetic ascorbic acid. The megaloblastic anemia observed in infants fed proprietary milk preparations has been explained as being due to a deficiency of folic acid secondary to chronic vitamin C deficiency. The leaves and flowers of the gladiolus, nettle, hip, and paprika are exceptionally rich in vitamin C. During World War II, rose hips, green walnuts, and wild cherries were used in Europe for the preparation of vitamin C concentrates.

Chemistry of Ascorbic Acid. Ascorbic acid crystallizes in white needles or plates having a melting point of 190° to 192° C. One g. dissolves in 3 ml. water, in 50 ml. absolute ethyl alcohol, and in 100 ml. glycerol. The vitamin is insoluble in benzene, ethyl ether, petroleum ether, and most organic solvents. Ascorbic acid possesses two asymmetric carbon atoms. The vitamin has a specific rotation $[\alpha]_D^{20} = +21°$ in water and +48° in methanol. D-Ascorbic acid, which differs from the L-isomer only in its configuration about the fourth carbon atom, is physiologically inactive. The acidic properties of ascorbic acid are due not to the carboxyl group, which is tied up in lactone form, but to ionization of the enol group on the third carbon atom. The vitamin is a comparatively strong acid, as indicated by the acidic dissociation constants, $pK_1 = 4.17$ and $pK_2 = 11.57$. A one-half per cent solution of ascorbic acid in water has a pH of approximately 3. At neutrality, the vitamin has an absorption maximum in the ultraviolet region of the spectrum at 265 mμ which shifts toward the shorter wavelengths with decreasing pH. Ascorbic acid is precipitated by lead ion at pH 7.6, but the salt can be redissolved in mineral acid at pH 2.

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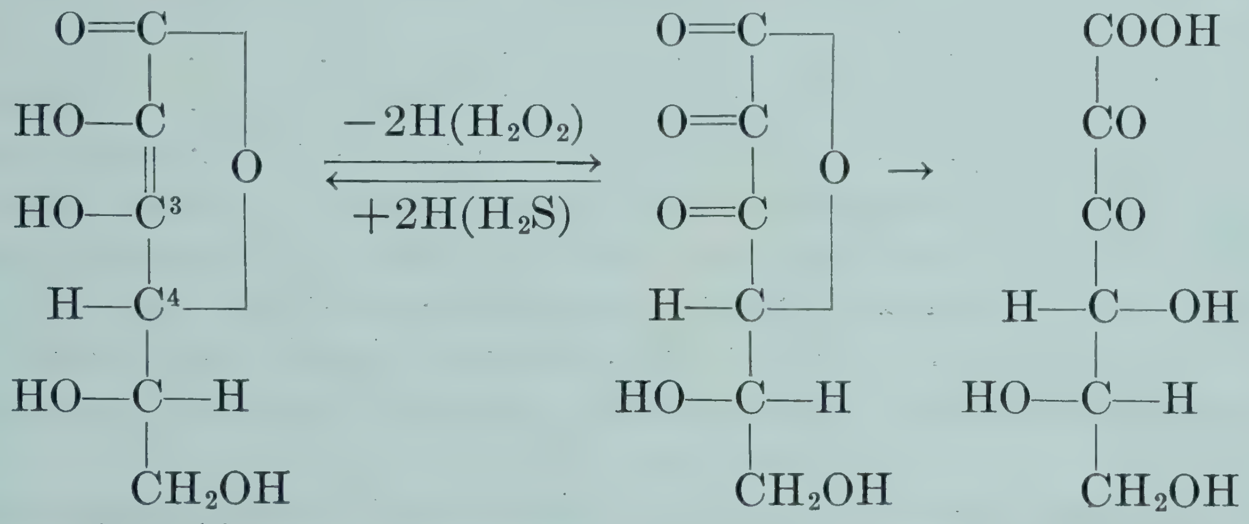
[324] For ascorbic acid values of foods, see Appendix III.

Ascorbic acid crystals are stable in air for years. In solution the vitamin is easily oxidized, the instability increasing with increasing pH. Mild oxidation such as that produced by air, hydrogen peroxide, ferric chloride, quinone, iodine in acid or neutral solutions, or 2,6-dichlorophenol indophenol converts the vitamin to dehydroascorbic acid as shown in the reaction below. Dehydroascorbic acid retains the biological activity of the vitamin and is reduced in animal tissues, a reaction in which sulfhydryl compounds like glutathione play an important part. The reduction of the dehydroascorbic acid may be accomplished *in vitro* by means of hydrogen sulfide. Above pH 5 dehydroascorbic acid readily undergoes a rearrangement in which the lactone ring is split. The product, diketogulonic acid, is no longer biologically active and is not reducible by hydrogen sulfide. Treatment with hydrogen iodide, however, converts this compound to dehydroascorbic acid, which may subsequently be reduced with hydrogen sulfide to ascorbic acid. Once the lactone ring has opened, the molecule readily undergoes further oxidation and may be degraded to oxalic acid. The oxidation of ascorbic acid by molecular oxygen is catalyzed by cupric and silver ions. Plant tissues contain several enzymes, including ascorbic acid oxidase, polyphenol oxidase,



L-Ascorbic acid **Dehydroascorbic acid** **Diketogulonic acid**

and peroxidase, which even more strongly catalyze the oxidation of the vitamin by molecular oxygen to dehydroascorbic acid. It is interesting to note that ascorbic acid oxidase is a copper-protein complex and that cupric ion in the presence of nonspecific protein is a stronger oxidation catalyst for vitamin C than cupric ion alone. Ascorbic acid is a strong reducing agent; its capacity to reduce silver nitrate, iodine, ferricyanide, methylene blue, and 2,6-dichlorophenol indophenol serves as the basis of methods for its histological detection and chemical estimation. The ease of oxidation of ascorbic acid is responsible for substantial losses during the processing of foods, which may be diminished by such measures as blanching (heat denaturation of enzymes), the use of an inert atmosphere (nitrogen or carbon dioxide), and storage at low temperatures. The ready solubility of ascorbic acid in water is also responsible for processing losses through extraction of the vitamin by the cooking water.

The destruction of ascorbic acid is catalyzed by light, especially in the presence of flavins. The vitamin reacts with niacin (or niacinamide) when the two are mixed in a thick paste, forming a colored compound. Ascorbic acid undergoes a similar reaction with other pyridine and quinoline compounds. The reaction is not attended by a decrease in the reducing properties of vitamin C. It has not been established whether any loss in the biological activity of either ascorbic acid or niacin occurs during the reaction.

Ascorbic acid exhibits optical specificity. The L-configuration about the fourth carbon atom is essential for biological activity, the D compound being biologically inactive. D-Araboascorbic acid (isoascorbic acid) which differs from L-ascorbic acid in the D configuration about the fifth carbon atom, possesses only one-twentieth of its activity. 6-Deoxy-L-ascorbic acid, which lacks the hydroxyl group on the sixth carbon atom, has one-fifth the activity of L-ascorbic acid. Other analogs of vitamin C, L-glucoascorbic acid, L-fucoascorbic acid, and D-glucoheptoascorbic acid have one-fortieth, one-fiftieth, and one-hundredth of the activity of vitamin C, respectively. However, the only naturally occurring forms of vitamin C are L-ascorbic acid and dehydroascorbic acid, both of which have equal biological activity. The sodium, copper, manganese, iron, monoethanolamine, and quinine salts of ascorbic acid are biologically active.

The synthesis of ascorbic acid can be effected by enolization of keto-hexonic acids or by condensation of lower aldehydes or of oxyesters. A commercial synthesis is represented by the following scheme:

$$\text{L-Sorbitol} \xrightarrow[\textit{B. xylinum}]{\text{Action of}} \text{L-Sorbose} \xrightarrow[+\mathrm{H_2SO_4}]{\text{Acetone}} \text{Diacetonesorbose} \xrightarrow{\text{Alk. }\mathrm{KMnO_4}} \text{2-Keto-L-}$$

$$\text{gulonic acid} \xrightarrow{\text{MeOH}} \text{Me ester of 2-keto-L-gulonic acid} \xrightarrow[\text{in MeOH}]{\text{HCl}} \text{L-Ascorbic acid}$$

Though the synthetic methods involving the 2-keto-hexonic acids are economically more practical, the preparation of vitamin C concentrates from natural materials received considerable attention in Europe during World War II.

DETERMINATION OF ASCORBIC ACID

Chemical methods for the determination of vitamin C are based, for the most part, upon the reducing properties of the vitamin. These procedures include titration of an acid extract with iodine, methylene blue in the presence of light, 2,6-dichlorophenol indophenol, or other oxidizing agents. Various acids have been used for the extraction, including acetic, trichloroacetic, metaphosphoric, and oxalic. The latter two acids serve not only to reduce the pH of the extracting medium, thereby stabilizing vitamin C, but also to form complexes with metallic ions, e.g., copper, thereby preventing the catalytic oxidation of the vitamin. Titration with an oxidizing agent is not a specific reaction: other reducing materials interfere, e.g., stannous and ferrous sulfate, sulfhydryl compounds, sulfides, thiosulfates, and reducing materials found in caramelized and fermented foods. Errors arise particularly in food products because of the presence of reductinic acid and reductones. Actually, reductone is hydroxypyruvic aldehyde, a compound formed by the alkaline hydrolysis of sugars. Reductinic acid is formed when pentoses are treated with acids. The terms reductone and reductinic acid, however, have been used rather loosely to signify other reducing compounds formed during heat-processing and storage of foods.

Numerous attempts have been made to increase the specificity of the oxidation-reduction methods, particularly that employing the 2,6-dichlorophenol indophenol dye. A number of these are based on the fact that ascorbic acid reacts very rapidly with the oxidizing agent whereas many of the interfering substances react more slowly. The simplest expedient for increasing the specificity is to conduct the titration very rapidly, though it presents little improvement over the original method. A more satisfactory procedure involves measurement of the rate of reaction by means of a photoelectric colorimeter. When observations are made five and ten seconds after mixing the ascorbic acid solution with the oxidant, and the values are extrapolated to "zero-time," the determination possesses sufficient specificity for vitamin C in most food products.

Modifications of the indophenol titration have been aimed particularly at eliminating the interference of reductones and reductinic acid. These compounds are similar to vitamin C in structure, stability, and chemical reaction. Like ascorbic acid, their oxidative destruction is catalyzed by copper and by ascorbic acid oxidase. A number of procedures for eliminating their interference involve condensation of the reducing substances with formaldehyde, which eliminates the reducing properties of ascorbic acid, reductones, and reductinic acid; but the interfering compounds may be differentiated from the vitamin by conducting the condensations at different pH levels.[325] There is no doubt that these procedures increase the specificity of indophenol titration, though the interference of reductone or reductinic acid is not completely eliminated. Moreover, errors arise because the procedures fail to take into account the presence of materials which affect the rate of combination of L-ascorbic acid with formaldehyde.[326]

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[325] Lugg: *Nature*, **150**, 577 (1942); *Australian J. Exptl. Biol. Med. Sci.*, **20**, 273 (1942); Mapson: *Nature*, **152**, 13 (1943).

One method of improving the specificity of the determination of vitamin C involves the destruction of ascorbic acid with ascorbic acid oxidase, thereby providing a blank correction value.[327] This procedure, however, is based on the assumption that the action of ascorbic acid oxidase is specific for vitamin C; whereas reductones are also destroyed.[328] Natural pigments and stubborn turbidities frequently interfere with the determination of vitamin C. Several modifications of the indophenol methods may eliminate these interferences since they involve extraction of the excess of unreacted dye with organic solvents (e.g. xylene) immiscible with water.[329]

Dehydroascorbic acid, the reversibly oxidized, biologically active form of vitamin C, does not possess reducing properties. Since this form of the vitamin is frequently present in considerable proportion in processed foods,[330] it must first be reduced before it can be determined with oxidizing agents. Hydrogen sulfide has been used most frequently for this purpose, but this reagent has been criticized on the ground that it creates other reducing materials which interfere with the procedure. This objection is valid when simple titrimetric methods are employed for the determination of vitamin C. However, little interference arises from this source when the photometric method involving measurements at five and ten seconds is employed. Other methods for reducing the dehydroascorbic acid have been suggested but are not widely employed. These include the reduction of the oxidized vitamin by a resting suspension of *E. coli*[331] and by electrolytic reduction.[332]

Dehydroascorbic acid and its inactive oxidation product, diketogulonic acid, couple with 2,4-dinitrophenylhydrazine to yield an osazone which gives a red color with strong sulfuric acid. By means of appropriate oxidation and reduction reactions, Roe and his associates[333] have adapted the dinitrophenylhydrazine reaction to the measurement of reduced and dehydroascorbic acids (both biologically active) as well as diketogulonic acid. This permits estimation of the extent to which the original vitamin C content of foods has undergone oxidation. Since fresh or well-preserved foods usually contain less than 5 per cent diketogulonic acid,[334] the dinitrophenylhydrazine method is usually used in its simpler form adapted to the measurement of only the biologically active forms of ascorbic acid. If oxidation during processing or storage is suspected, failure to account for diketogulonic acid may lead to serious errors.[335,336]

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[326] Snow and Zilva: *Biochem. J.*, **37**, 630 (1943).

[327] Tauber and Kleiner: *J. Biol. Chem.*, **110**, 559 (1935).

[328] Snow and Zilva: *Biochem. J.*, **32**, 1926 (1938); Wacholder and Okrent: *Z. physiol. Chem.*, **264**, 254 (1940); Stewart and Sharp: *Ind. Eng. Chem., Anal. Ed.*, **17**, 373 (1945).

[329] Bukatsch: *Z. physiol. Chem.*, **262**, 20 (1939). See also Pepkowitz: *J. Biol. Chem.*, **151**, 405 (1943). The possibility of error due to the reaction of anthocyanins with the dye is emphasized by Somers, *et al.*: *Science*, **110**, 17 (1949).

[330] Hochberg, Melnick, and Oser: *Ind. Eng. Chem., Anal. Ed.*, **15**, 182 (1943); McMillan and Todhunter: *Science*, **103**, 196 (1946).

[331] Gunsalus and Hand: *J. Biol. Chem.*, **141**, 853 (1941).

[332] Gunther: *Biochem. Z.*, **314**, 277 (1943).

[333] Roe and Kuether: *J. Biol. Chem.*, **147**, 399 (1943); Roe and Oesterling: *Ibid.*, **152**, 511 (1944); Roe, *et al.*: *Ibid.*, **174**, 201 (1948).

[334] Mills, Damron, and Roe: *Anal. Chem.*, **21**, 707 (1949).

The determination of ascorbic acid in blood and urine is employed in the diagnosis of clinical and subclinical vitamin C deficiency. These values fluctuate widely even in normal individuals depending upon the ascorbic acid intake just prior to the test. For this reason blood tests are conducted preferably when the subject is in a fasting state. A serum or plasma level greater than 0.7 mg. per 100 ml. is regarded as normal whereas values less than 0.4 mg. are observed in scurvy. Some clinicians set 1.2 mg. per 100 ml. as the minimum normal level. Individuals in a normal state of nutrition excrete from 20 to 50 mg. of ascorbic acid when fed a diet containing 100 mg. per day. Since both blood levels and urinary excretion values depend upon the ascorbic acid intake for one or two days prior to the test, a more satisfactory index of ascorbic acid nutrition may be obtained by measurement of the response of the subject to a large dose (500 to 1000 mg.) of ascorbic acid. Saturated subjects excrete approximately 25 to 50 per cent of the test dose in 24 hours, and show a continued high blood level for hours following dosage. In one saturation test, an excretion of 20 mg. of ascorbic acid in the four-hour period following the ingestion of 200 mg. is regarded as normal.

Plasma ascorbic acid values fall very rapidly when a deficient diet is consumed, though tissue stores including those of blood cells have not yet become depleted. It has been suggested that the ascorbic acid content of the leukocytes or of whole blood[337] is a better index of the state of vitamin C nutrition of the tissues than the blood plasma level. Oxyhemoglobin interferes with the determination of ascorbic acid in whole blood by oxidation-reduction procedures unless it is saturated with carbon monoxide[337] or reduced.[338] The 2,4-dinitrophenylhydrazine method (see p. 1237) is suitable for determination of ascorbic acid in whole blood.

One procedure intended for clinical diagnosis of ascorbic acid deficiency is based on intradermal injection of 2,6-dichlorophenol indophenol followed by measurement of the rate of decolorization of the dye.[339]

Modified Titrimetric Method of Bessey:[340] ***Principle.*** The method depends on the stoichiometric reduction of the dye 2,6-dichlorophenol indophenol to a colorless compound by ascorbic acid. The titration is conducted in the presence of acetic and metaphosphoric acids in order to inhibit aerobic oxidation catalyzed by certain metallic ions, to inactivate enzymes, and to precipitate proteins and liberate protein-bound ascorbic acid. A modification of this method is based on the addition of an excess of the dye and measurement of the unreacted portion. In the presence of interfering pigmentation or turbidity, the dye can be removed by xylene extraction and read in a photometer.[341] It is necessary however, to guard against the extraction of xylene-soluble pigments present in some heat-processed foods, and certain non-vitamin-C substances (e.g., betanin of beets) which react with the dye.

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[335] Penney and Zilva: *Biochem. J.*, **37**, 39 (1943).

[336] Pijoan and Gerjovich: *Science*, **103**, 202 (1946).

[337] Butler and Cushman: *J. Clin. Investigation*, **19**, 459 (1940); Roe and Kuether: *loc. cit.*

[338] Kuether and Roe: *Proc. Soc. Exptl. Biol. Med.*, **47**, 487 (1941).

[339] Reddy and Sastry: *Indian Med. Gaz.*, **76**, 476 (1941).

[340] Bessey: *J. Assoc. Official Agr. Chem.*, **27**, 537 (1944).

[341] See footnote 329, p. 1234.

Procedure. **Macerate an aliquot of the sample containing 5 to 50 mg. ascorbic acid with 150 ml. metaphosphoric-acetic acid solution[342] in a Waring Blendor. Dilute to 200 ml. and filter. Titrate a 10- to 100-ml. aliquot with standard indophenol solution.**

Preparation of Standard Indophenol Solution. **Dissolve 42 mg. sodium bicarbonate and 52 mg. sodium 2,6-dichlorophenol indophenol in 50 ml. water. Dilute to 200 ml. Filter and store in the refrigerator not more than three days. Dissolve 100 mg. crystalline ascorbic acid in 100 ml. metaphosphoric acetic acid solution. Dilute a 10-ml. aliquot with 25 ml. metaphosphoric-acetic acid solution, and titrate with indophenol solution until the pink color persists for 5 seconds. Calculate and express the strength of the indophenol solution as mg. ascorbic acid equivalent per ml. reagent. Repeat the standardization each day with a freshly prepared standard ascorbic acid solution.**

CALCULATION. Calculate the ascorbic acid content of the sample using the formula,

$$V \times S \times D = \text{mg. ascorbic acid per g. of sample}$$

where V is ml. of dye used to titrate the unknown, S is the standardization value expressed in mg. ascorbic acid per ml., and D is the dilution factor.

Interpretation. The simple titrimetric method is applicable to the determination of ascorbic acid only in the absence of other reducing substances, and where only the reduced form of the vitamin is present. Thus it may be applied to fresh orange, grapefruit, lemon, lime, or tomato juice and to some pharmaceutical preparations. For heat-processed materials and other foods, it is necessary to eliminate interfering substances and to determine the reversibly oxidized, biologically active form of the vitamin, dehydroascorbic acid. Ferrous ion reduces the dye in the presence of metaphosphoric acid. Pharmaceutical preparations containing reduced iron may be titrated in 8 per cent acetic acid solution free from metaphosphoric acid. On the other hand, ferric ion interferes with the end point in the absence of metaphosphoric acid; hence the metaphosphoric-acetic acid mixture should be employed as the titration medium when testing pharmaceutical preparations containing oxidized iron.[343]

Determination of Ascorbic Acid in Plant Tissues (Modified Colorimetric Method of Roe and Kuether):[344] ***Principle.*** Ascorbic acid is oxidized to dehydroascorbic acid by shaking with "Norit" in the presence of acetic acid. After coupling with 2,4-dinitrophenylhydrazine, the solution is treated with sulfuric acid to produce a red color which is measured photometrically.

Procedure. **Grind 10 g. of sample in sufficient 5 per cent metaphosphoric-10 per cent acetic acid solution to produce a concentration of 5–15 μg. ascorbic acid per ml. Filter. To a 15-ml. aliquot of the clear filtrate, add 0.75 g. acid-washed "Norit"[345] and shake vigorously. Filter. To a 4-ml. aliquot of the**

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[342] Dissolve 15 g. of stick metaphosphoric acid in a mixture containing 40 ml. glacial acetic acid and 450 ml. water. Filter, and store in the refrigerator. Discard after 10 days.

[343] Gawron and Berg: *Ind. Eng. Chem., Anal. Ed.*, 16, 757 (1944).

[344] Roe and Kuether: *loc. cit.;* Roe and Oesterling: *loc. cit.*

[345] Suspend 100 g. "Norit" in 500 ml. 10 per cent hydrochloric acid. Heat to boiling, then filter with suction. Remove the cake of "Norit," stir it up with 500 ml. of water, and filter again. Repeat this procedure until the washings give a negative or faint test for ferric ions. Dry overnight in an oven at 110°–120° C.

filtrate in a test tube, add 1 drop 10 per cent thiourea solution, and 1 ml. 2,4-dinitrophenylhydrazine reagent.[346] Place the tube in a water bath maintained at 37° C. for exactly 3 hours. Remove and place in an ice-water bath along with a blank tube containing 4 ml. of "Norit" filtrate and 1 drop thiourea solution. To each tube in the bath add dropwise 5 ml. 85 per cent sulfuric acid[347] with stirring. Finally add 1 ml. 2,4-dinitrophenylhydrazine reagent to the blank. Remove the tubes from the bath and allow to stand 30 minutes. Read the colors in a photoelectric colorimeter with a filter transmitting maximally at 540 mμ, setting the instrument at 100 per cent transmittance with the blank tube.

CALCULATION. Prepare a calibration curve by testing 4-ml. aliquots of appropriate standards containing 0.25–15.0 μg. ascorbic acid per ml. carried through the entire procedure. Plot photometric density against μg. ascorbic acid per ml. From the curve, estimate the ascorbic acid concentration of the "Norit" filtrate of the unknown and multiply this by the dilution factor to obtain the ascorbic acid content of the sample.

Interpretation. The 2,4-dinitrophenylhydrazine procedure measures total ascorbic acid. This may be partitioned into the reduced and dehydro forms by including a simultaneous test in which the "Norit" treatment is omitted. The latter procedure measures only dehydroascorbic acid. The reduced ascorbic acid content is obtained by difference. Reductones, degradation products of sugars, and diketogulonic acid interfere with the determination of ascorbic acid by this procedure.

Determination of Ascorbic Acid in Whole Blood, Plasma, or Urine: Method of Roe and Kuether.[348]

Procedure. **Add 5 ml. whole blood or plasma, dropwise with stirring, to 15 ml. 6 per cent trichloroacetic acid in a 50-ml. centrifuge tube. Stir to obtain a fine suspension. Allow to stand 5 minutes, then centrifuge. Add 0.75 g. acid-washed "Norit" (see above) to the clear supernatant solution, and shake vigorously. Filter. Test 4-ml. aliquots by the 2,4-dinitrophenylhydrazine procedure as directed above.**

To 2 ml. urine, add 38 ml. 4 per cent trichloroacetic acid solution. (A greater dilution should be made if the sample is expected to contain more than 300 mg. ascorbic acid per liter.) Add 1.5 g. acid-washed "Norit" (see above), shake vigorously, and filter. Test 4-ml. aliquots by the 2,4-dinitrophenylhydrazine procedure as directed above.

Photometric Method of Hochberg, Melnick, and Oser:[349] Principle. Ascorbic acid is determined in the presence of other reducing substances by photometric measurement of the rate of decolorization of the dye 2,6-dichlorophenol indophenol. Dehydroascorbic acid is determined after reduction by hydrogen sulfide.

Procedure. **Conduct all manipulations under an atmosphere of nitrogen and make all extractions and dilutions with solutions previously deaerated with a stream of nitrogen. Homogenize the sample with an equal weight of 6 per cent metaphosphoric acid solution.[350] (Fresh vegetables and materials**

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[346] Dissolve 2 g. 2,4-dinitrophenylhydrazine in 100 ml. 9 N sulfuric acid and filter.

[347] To 100 ml. distilled water, add 900 ml. concentrated sulfuric acid, sp. gr. 1.84.

[348] Roe and Kuether: *loc. cit.*

[349] Hochberg, Melnick, and Oser: *Ind. Eng. Chem., Anal. Ed.*, 15, 182 (1943).

[350] Metaphosphoric acid changes slowly in solution to orthophosphoric acid. The extractant should be stored in the refrigerator where it is stable for several weeks.

containing active oxidases should first be blanched by adding the sample directly to boiling 6 per cent metaphosphoric acid. After boiling for 5 minutes, cool and homogenize.) Dilute to a convenient volume with 3 per cent metaphosphoric acid. Shake mechanically for 15 minutes, then centrifuge. To 50 ml. of the clear supernatant fluid, add 14 ml. of citrate solution.[351] Measure reduced ascorbic acid in this aliquot as directed below.

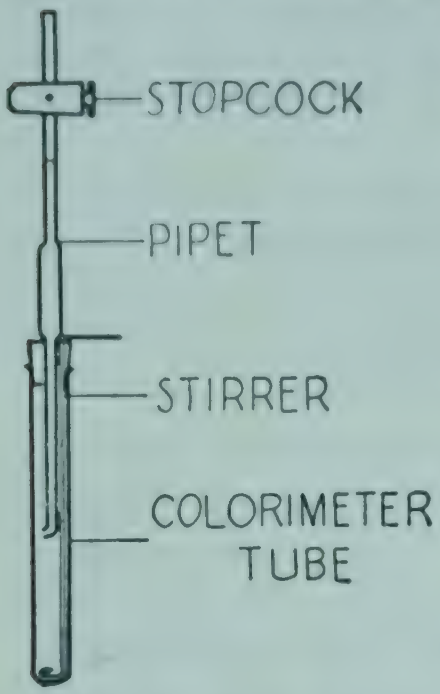

FIG. 284. PIPET USED FOR ADDITION OF ASCORBIC ACID EXTRACT TO DYE SOLUTION.

Convert dehydroascorbic acid to the reduced form by treating the metaphosphoric citric acid extract with a slow stream of hydrogen sulfide for 20 minutes. Allow to stand for 2 hours, then flush out the hydrogen sulfide with a vigorous stream of wet nitrogen (bubbled through water) for 2 hours.

Colorimetric Measurement. **Determine the volume of extract necessary to decolorize 15 ml. 2,6-dichlorophenol indophenol solution.[352] To obtain optimal concentration for measurement in a photoelectric colorimeter, dilute this volume of extract to 30 ml. with metaphosphoric-citric acid solution.[353]**

Place a colorimeter tube containing 5 ml. of standard dye solution in the instrument. Add 5 ml. of the diluted extract from a rapid delivery pipet[354] (Fig. 284) and measure the residual photometric density[355] 5 and 10 seconds later. Determine the blank absorption of extraneous pigments in the extract by adding a few crystals of ascorbic acid to completely decolorize the residual dye.

Obtain the residual photometric density at 5 and 10 seconds with standard solutions of ascorbic acid containing 1 to 6 μg. per ml. in metaphosphoric citric acid solution.

CALCULATION. Plot the photometric densities for the standard solutions (ordinates) against time. Extrapolate the readings linearly to zero time. This calibrates the ordinates in terms of ascorbic acid concentration.

Calculate the ascorbic acid concentration of the unknown as follows: Subtract the photometric density of the blank from those at 5 and 10 seconds. Plot the values as above and extrapolate to zero time. Estimate the ascorbic acid concentration of the extract from the intercept on the vertical axis, calibrated as described above. Multiply by the dilution factor to obtain the ascorbic acid content of the original sample.

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[351] *Citrate Solution.* Dissolve 21 g. citric acid in 200 ml. 1.0 N sodium hydroxide and dilute to 250 ml.

[352] *2,6-Dichlorophenol Indophenol Solution.* Dissolve 8.0 mg. 2,6-dichlorophenol indophenol sodium salt in hot water and filter. Wash the filter paper several times with cold water and dilute the combined filtrate and washings with distilled water so that 5 ml. dye plus 5 ml. metaphosphoric-citric acid transmit 40 per cent of the incident light in the photoelectric colorimeter. The final volume is approximately 500 ml. Set the instrument at 100 per cent transmittance with distilled water.

[353] *Metaphosphoric-Citric Acid Solution.* Add 70 ml. citrate solution to 250 ml. 3 per cent metaphosphoric acid solution.

[354] The pipet should deliver exactly 5 ml. of extract in a period of less than one second. To prevent formation of air bubbles, the solution should be delivered against the side of the tube.

[355] The photoelectric colorimeter must be of the direct reading (not null-point) type and its galvanometer must be rapid and critically damped. Set the instrument at 100 per cent transmittancy with distilled water. The concentrations of ascorbic acid and dye and the volumes employed are those recommended when the Evelyn photoelectric colorimeter (Rubicon Company, Philadelphia) is employed.

Interpretation. This photometric method measures both reduced and dehydroascorbic acid. Appreciable concentrations of the latter are frequently found in processed and stored foods. The method is applicable to the determination of ascorbic acid in the presence of other reducing materials, which interfere minimally in this procedure.

BIOLOGICAL METHODS

Because of its nondependence on a dietary source of ascorbic acid, the rat cannot be used for vitamin C assay. The animal employed is the guinea pig, since it is extremely sensitive to the lack of this factor in the diet. The techniques used are based either on the determination of the minimum dose necessary for protection against or cure of gross scorbutic symptoms,[356] or on the histological examination of the incisor teeth[357] after a test period of 14 days. The latter method requires equipment not always available in nutrition laboratories, though it has proved satisfactory for the determination of minimum protective dose where large enough groups are used to compensate for variations encountered. About twice as much ascorbic acid is required for protection of the dental pulp of guinea pigs as for prevention of gross scurvy.[358] The histological method has the advantages of high specificity and a short test period. Nevertheless, because of the special facilities required it has not been as widely used as the procedures based on prophylaxis or cure of gross symptoms.

The minimum daily allowance of a food which provides a guinea pig with complete protection from scurvy is a measure of its vitamin C potency. It has been suggested by Sherman, La Mer, and Campbell that by rating the pathological effects observed in the living animal and at autopsy, the fraction of complete protection afforded by subminimal doses may be estimated. While this method allows more animals to be considered in interpreting a given experiment, considerable experience is required in diagnosing and scoring the extent of the lesions.

ASSAY FOR VITAMIN C

Biological Method of Sherman, La Mer and Campbell. **Guinea pigs, 6 to 8 weeks old and weighing from 250 to 300 g., are fed *ad lib.* the following scurvy-producing diet:**[359]

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[356] Sherman, La Mer, and Campbell: *J. Am. Chem. Soc.*, **44**, 165 (1922); Coward and Kassner: *Biochem. J.*, **30**, 1719 (1936).

[357] Höjer: *Brit. J. Exptl. Path.*, **7**, 356 (1926); Goettsch: *Quart. J. Pharm.*, **1**, 168 (1928); Key and Elphick: *Biochem. J.*, **25**, 888 (1931); Fish and Harris: *Proc. Roy. Soc. London*, **223**, 489 (1933).

[358] Eddy: *Am. J. Pub. Health*, **19**, 1309 (1929).

[359] Diets containing fat must be prepared fresh weekly, as guinea pigs reject rancid food. Other scorbutigenic diets which have proved useful are:

(*a*) *Cambridge Nutrition Laboratory* (Harris *et al.*). Bran 80, oats 720, dried egg yolk 40, salts 8.4, codliver oil 1.

(*b*) Demole: *Z. Vitaminforsch.*, **3**, 89 (1934). Rolled oats 2 kg., dried whole milk 1 kg. (previously dried at 120° C. for 2 hr.). Mix and make into paste with whites of 6 eggs and water. Mold into cakes 5 cm. diam. and bake 20–25 min. on a tin greased with olive oil. Supplement with 0.2 ml. codliver oil weekly.

(*c*) *Purina Rabbit Chow* (Purina Mills, St. Louis, Mo.) produces scurvy in guinea pigs unless supplemented with a source of ascorbic acid.

Ground whole oats[360]	59
Heated nonfat milk solids[361]	30
Fresh butterfat[362]	10
Salt	1

This diet may be reinforced with yeast and codliver oil.

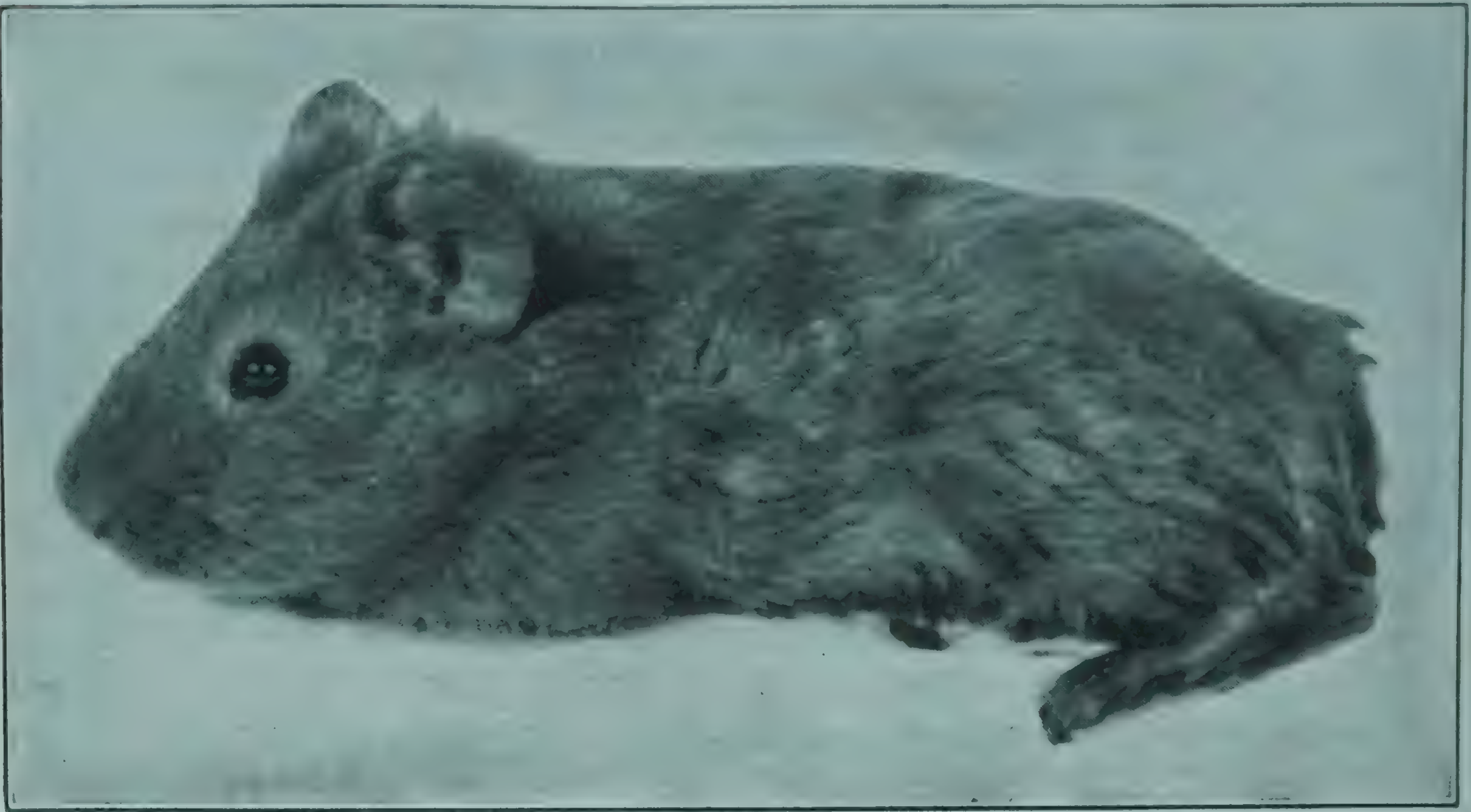

FIG. 285. SCORBUTIC GUINEA PIG, SHOWING "SCURVY POSITION."

In order to provide assurance of the health of the guinea pigs and their capacity to grow, a short preliminary period may be included during which some source of vitamin C is fed, such as a leafy vegetable or orange juice. Following this period the food to be assayed is fed in graded doses as the sole source of vitamin C. Feed 0.5 ml. 0.1 per cent ascorbic acid or 5 ml. orange or lemon juice to positive control animals as a supplement.

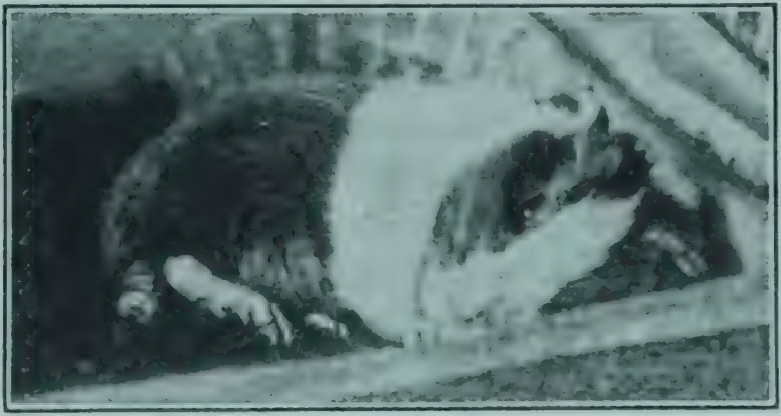

FIG. 286. GUINEA PIG WITH SCURVY, SHOWING "FACE-ACHE POSITION."

Special Report of British Medical Research Committee, No. 38, 1919.

In the absence of antiscorbutic vitamin, growth ceases in about 2 weeks, and a rapid decline in weight is followed by death in 4 to 5 weeks. During the second or third week, the external symptoms of scurvy become evident.[363] The joints become tender, causing the guinea pig to wince or squeal when pressure is applied to the wrists. The paws become edematous and hemorrhagic. The animal becomes lethargic instead of excitable, and usually assumes an unnatural position such as holding up a tender hind leg (the "scurvy position," Fig. 285) or lying with the side of its face resting on the floor (the "face-ache position," Fig. 286). The characteristic postmortem findings are apt to be more pronounced when the deficiency is not quite complete, because the lesions have time to reach an exaggerated state. These findings include hemorrhages (which may be subcutaneous, intramuscular, or intraabdominal), loosening of the teeth, fragility of the bones and teeth, and enlargement of the costochondral junctions of the ribs (beading).

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[360] A mixture of equal parts of commercial rolled oats and wheat bran may be used instead of the ground whole oats.

[361] Heated in shallow open pans at 110° C. until all the vitamin C is destroyed, as determined by chemical assay. The powder assumes a brownish color.

[362] The butterfat may be replaced by vegetable oil 8 + codliver oil 2.

[363] The symptomatology of scurvy in guinea pigs is fully described by Cohen and Mendel: *J. Biol. Chem.*, 35, 425 (1918).

Supplying a source of vitamin C to a guinea pig in even an advanced state of scurvy usually will bring about prompt recovery, unless intercurrent disease has set in. About 0.5–0.6 mg. is required daily for complete protection or cure.

The vitamin C content is calculated from the minimum daily allowance which affords complete protection from scurvy to a standard guinea pig, as above described. Comparison of this dose with the corresponding allowance of the crystalline L-ascorbic acid enables one to express the result of the bioassay on a mg. basis instead of in now obsolete International Units. The International Standard is crystalline L-ascorbic acid, of which 0.05 mg. equals one International Unit of vitamin C.

BIOFLAVONOIDS

In 1936, Szent-György[364] prepared an extract from paprika and subsequently from citrus juice, which he claimed to have value beyond that of ascorbic acid in reducing capillary bleeding in man and in guinea pigs. The name vitamin P was applied to this factor in the belief that it was a dietary factor necessary for the maintenance of normal capillary permeability. Interest in various hemorrhagic conditions which do not respond to vitamin K or ascorbic acid has led to widespread investigation of concentrates high in the active permeability factor.

Physiological and Clinical Aspects of Bioflavonoids. Although the literature is confusing on this point, a distinction should be drawn between capillary fragility caused by a change in the structural integrity of the capillary walls (e.g., loss of intercellular cement substance as in scurvy) and increased permeability without apparent tissue damage. Clear differentiation is not possible in the usual tests based on subcutaneous petechial hemorrhage induced by negative pressure.

Cumulated evidence has demonstrated the existence of a considerable number of flavonoid or related compounds having the protective effect on capillary resistance associated with vitamin P activity. Some of the preparations used in animal and clinical studies, e.g., citrin, are actually mixtures of only partially identified compounds. Effective doses vary over a wide range. In view of such considerations it is believed that the activity of the flavonoids is a pharmacological one rather than a specific physiological function for which a dietary factor is essential. Since continued application of the term "vitamin P" to one or another of these polyphenolic substances would lead only to confusion, it was recommended[365] that the term be no longer employed. The term "bioflavonoids" has been proposed for this class of compounds.[366]

Scarborough[367] describes the characteristic syndrome of petechial bleeding as accompanied by pain across the shoulders and in the legs, lassitude and undue fatigue, low capillary resistance, slightly prolonged bleeding time, and low serum calcium. The bioflavonoids are effective in relieving this syndrome but pure ascorbic acid is not.

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[364] Szent-György and Rusznyák: *Nature*, **138**, 27 (1936).

[365] Joint Committee on Nomenclature, American Society of Biological Chemists and American Institute of Nutrition: *Science*, **112**, 628 (1950).

[366] Oser: cited by Bryant: *J. Am. Pharm. Assoc.*, **39**, 480 (1950).

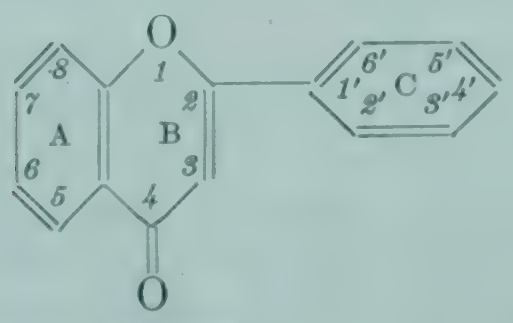
[367] Scarborough, *et al.*: *Biochem. J.*, **33**, 1400 (1939); *Lancet* **2**, 610 (1938); 644 (1940); *Edinburgh Med. J.*, **50**, 85 (1943).

It has been postulated by the French workers[368] that epinephrine is the compound directly responsible for the maintenance of capillary integrity and that the protective action of the bioflavonoids is an indirect one, based on their inhibitory effect on the oxidation of epinephrine (demonstrable both *in vivo* and *in vitro*). Certain flavonoids have been shown to produce a fall in arterial blood pressure in animals[369] and to inhibit hyaluronidase[370] and histidine decarboxylase,[371] but whether these effects are causally related to capillary resistance, and how, have not been established.

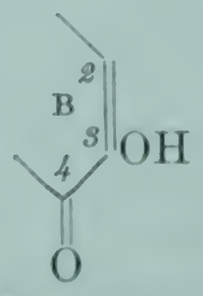
There is conflicting evidence in the literature regarding the clinical value of these compounds, which probably results from the fact that there are many factors responsible for the maintenance of normal capillary resistance. Clinical methods of measuring capillary fragility are inadequate because of diurnal variations and differences in subcutaneous capillary beds in different areas of the body. The dosages employed clinically depend upon the purity of the extracted flavones; the crystalline glycosides have been administered in divided dosage in the order of 50 to 150 mg. per day.

Occurrence of the Bioflavonoids. Citrin or similar glycosides having bioflavonoid activity have been isolated from paprika, juice or peel of citrus fruits, buckwheat, black currants, cured tobacco, and the leaves of a wide variety of plants.

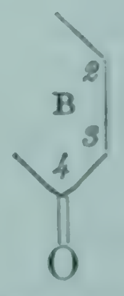
Chemistry of the Bioflavonoids. Most if not all of the active flavonoids occur in nature as glycosides, either alone or in combination. Citrin from lemon peel is said to be a mixture of the flavonone glycosides, hesperidin and eriodictin (the corresponding aglycones being hesperetin and eriodictyol, respectively) although its exact composition has not been clearly established. The relation of flavanones and flavonols to the parent nucleus of flavone (2-phenyl-1,4-benzopyrone) is shown by the following structural formulas.



Flavone



Flavonol



Flavanone

The chemical relationship of the aglycones and glycosides to each other is shown in the following tabulation.

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[368] Javillier and Lavollay: *Helv. Chim. Acta*, **29**, 1283 (1946).

[369] Higby: *J. Am. Pharm. Assoc.*, **32**, 74 (1943).

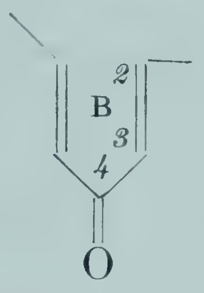
[370] Beiler and Martin: *J. Biol. Chem.*, **171**, 507 (1947).

[371] Martin, *et al.*: *Arch. Biochem.*, **21**, 177 (1949).

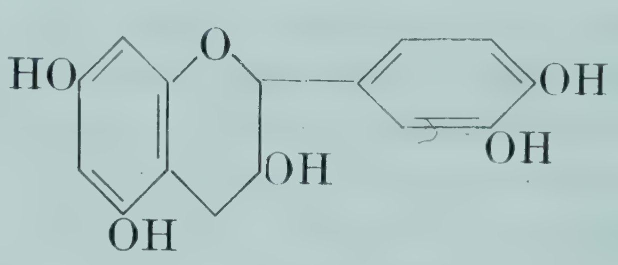
Flavonoids	*Nucleus*	*Substituents*	*Positions*
Eriodictyol*	Flavanone	4 (OH)	5, 7, 3′, 4′
Hesperetin*	Flavanone	3 (OH)	5, 7, 3′
		1 (OCH_3)	4′
Hesperidin	Flavanone	2 (OH)	5, 3′
		1 (OCH_3)	4′
		Glucorhamnose	7
Rutin	Flavonol	4 (OH)	5, 7, 3′, 4′
		Glucorhamnose	3
Quercetin*	Flavonol	4 (OH)	5, 7, 3′, 4′
Quercetrin	Flavonol	4 (OH)	5, 7, 3′, 4′
		Rhamnose	3

* Aglycones.

Certain chalcones, i.e., compounds resulting from the opening of the pyrone ring at the 1-2 position, are active, as are also catechin (not to be confused with the dihydric phenol catechol) and its stereoisomer *epicatechin*.



Opened ring of chalcone nucleus (cf. Flavone)



Catechin

Determination of Bioflavonoids. The varied chemical structure of naturally occurring bioflavonoids and their active derivatives has militated against the possibility of a chemical assay correlated with the specific type of biological activity. However analytical methods have been described for flavonoids in certain plant or animal tissues. The biological assay is based upon the observation of petechial hemorrhages under controlled conditions similar to the procedure employed for the diagnosis of "vitamin P" deficiency in man. Bacharach, Coates, and Middleton[372] have described a guinea pig assay in which crystalline hesperidin is used as the reference standard. The critical petechial pressure is determined from the negative pressure required to produce hemorrhages when a suction cup, furnished with a glass window for visibility, is applied to the prepared area of skin.

VITAMIN D

Early in the history of vitamins, antirachitic activity was ascribed to fat-soluble vitamin A, but the work of McCollum and his collaborators[373] established the distinction between the growth-promoting, antiophthalmic vitamin A and the antirachitic factor to which the name vitamin D was assigned. The specific role of this vitamin in the prevention and cure of rickets (rachitis) is integrated with the metabolism of calcium and phosphorus; in fact, in some species, e.g., the rat, the absence of vitamin D from the diet does not result in rickets unless the normal dietary ratio of Ca:P is disturbed. A condition analogous to human rickets may be produced experimentally in this species by feeding a diet high in calcium, low in phosphorus, and free from vitamin D. On a low-calcium, high-phosphorus diet, the bones develop an osteoporotic condition resembling osteomalacia. The function of vitamin D appears to be to mobilize these calcifying elements, so as to make possible their most efficient utilization even when the dietary supply is inadequate or disproportionate. It has been shown that even if calcium and phosphorus are fed in proper quantity and ratio, the absence of vitamin D, especially if prolonged, will result in poorer calcification of the bones than obtains under normal conditions.[374]

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[372] Bacharach, Coates, and Middleton: *Biochem. J.*, **36**, 407 (1942); See also Bourne: *Nature*, **152**, 659 (1943).

[373] McCollum, *et al.*: *J. Biol. Chem.*, **50**, 5 (1922); **51**, 41 (1922); **53**, 293 (1922).

Mild cases of rickets are often unrecognized, though they are quite prevalent, and may not have serious consequences until later years. The work of Jeans and his associates has emphasized that the requirement of vitamin D for optimum growth exceeds the antirachitic requirement. Rickets in its more severe manifestations is seen more frequently among urban children of poor economic status. In regions where sunshine is prevalent and customs permit bodily exposure, the disease is of rare occurrence. In India rickets is less common among the poor who live an outdoor existence than among the wealthy who observe the rite of seclusion (*purdah*).

Physiological and Clinical Aspects of Vitamin D. Rachitic bones are characterized by a lower content of mineral matter and an apparent overgrowth of osteoid cartilage, in consequence of which normal rigidity is absent. The sequelae of this condition in children are misshapen bones (e.g., bowlegs), epiphyseal enlargement (e.g., knock-knees and beading of the ribs, the "rachitic rosary"), delayed closure of the fontanelle, retarded eruption of the teeth, disturbances of respiration due to deformity of the thoracic cavity (pigeon breast), etc. In later life, difficult childbirth may be encountered because of pelvic deformities. The changes in bone composition are illustrated in the table on p. 1245.

The calcium phosphate compound in all bones, normal or rachitic, appears to be $Ca_3(PO_4)_2$, which in the normal adult rat is accompanied by $CaCO_3$ to the extent of about 15 per cent of the total calcium.[375] The ratio of tertiary phosphate to carbonate is reduced in the rachitic animal. From evidence based partly on solubility product studies, it has been suggested that primary calcification differs in composition from old

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[374] Sherman and Stiebeling: *J. Biol. Chem.*, **83**, 497 (1929); **88**, 683 (1930); *Proc. Soc. Exptl. Biol. Med.*, **27**, 663 (1930); Fairhall: *Am. J. Physiol.*, **84**, 378 (1928).

[375] Howland, Marriott, and Kramer: *J. Biol. Chem.*, **68**, 721 (1926). For a fuller discussion of the composition of bone see Chapter 9.

COMPOSITION OF NORMAL AND RACHITIC BONES (SCHABAD)[376]
(IN PER CENT OF DRY FAT-FREE MATTER)

Bone		*Water*	*Ash*	*Organic Matter*	*Calcium*	*Phosphorus*
Rib......	Normal	14.4–32.9	40.2–46.6	26.9–39.1	15.5–18.1	5.4–8.3
	Rachitic	42.4–66.4	7.9–32.0	20.7–22.4	3.0–12.0	1.4–5.6
Occiput..	Normal	13.0–16.1	47.6–51.7	32.2–36.5	18.8–19.9	7.9–9.0
	Rachitic	29.0–35.9	34.3–40.6	26.1–31.6	13.6–17.2	6.0–7.8

bone.[377] Refractive index and x-ray studies show that the solid inorganic phase of bone consists of minute crystals which are normally oriented lengthwise along the bone, but in rickets the cement substance (collagen) is broken down and the x-ray diffraction pattern indicates a disoriented crystalline structure.[378]

Many suggestive facts are known, but no complete explanation of the mechanism of vitamin D activity is available. The diminished retention and altered paths of elimination of calcium and phosphorus in rickets (illustrated in the table on p. 1246) provide the basis for the conception that vitamin D acts by promoting the absorption of calcium and phosphorus from the small intestine. A causal relation between the intestinal pH (probably only in the cecum and colon) and the retention of the calcifying elements is suggested by the greater alkalinity (and hence the precipitation of calcium salts) in rickets, but it is not known which of these is the determining factor and, if it is the former, what ultimate mechanism is responsible for its alteration in rickets. While the effect seems to be mediated through an increase in permeability, vitamin D does not appear to influence the excretion of calcium into the intestine, except indirectly. Vitamin D is claimed to increase gastric secretion and thus facilitate calcium absorption. The sharper peaked blood sugar tolerance curve after vitamin D administration is also suggestive of an effect on intestinal absorption.

A significant agent in ossification is the enzyme alkaline phosphatase.[379] This enzyme occurs in bone, ossifying cartilage, the kidneys and intestinal mucosa, and in low concentration (5 to 15 units per 100 ml.) in blood. By hydrolyzing phosphoric acid esters in bone it liberates inorganic phosphate which modifies the calcium and phosphate ion concentrations to the extent that the solubility product is exceeded and excess calcium phosphate is deposited. The optimum pH range of alkaline phosphatase is 8.4 to 9.4.

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[376] Prepared by Orgler (*Ergebnisse inn. Med. Kinderheilk.*, **8**, 142 (1912)) from the data of Schabad (*Arch. Kinderheilk.*, **52**, 47 (1909); **53**, 380 (1910); **54**, 83 (1911)) and cited by Hess (see Bibliography, p. 1296).

[377] Kramer and Shear: *Proc. Soc. Exptl. Biol. Med.*, **25**, 141 and 285 (1927–28).

[378] Taylor and Sheard: *J. Biol. Chem.*, **81**, 479 (1929). Clark and Mrgudich: *Am. J. Physiol.*, **108**, 74 (1934).

[379] Robison, *et al.*: *Biochem. J.*, **17**, 286 (1923); **18**, 740 and 1354 (1924); **19**, 153 (1925); **20**, 847 (1926); **23**, 767 (1929); **24**, 1922 and 1927 (1930); Kay: *Biochem. J.*, **20**, 791 (1926); **22**, 855 (1928); *J. Biol. Chem.*, **89**, 235 and 249 (1930).

The fact that phosphatase is concentrated in the ossifying centers of bone cartilage, even in rickets, would tend to indicate that in this disease the supply of substrate may be at fault. In rickets there is not only overproduction of osteoid matrix but also proliferation of cartilage cells which are an abundant source of phosphatase. The small amount of this enzyme normally found in plasma is greatly increased in generalized bone disorders, probably by diffusion from the osseous tissue (see p. 253) in which a compensatory stimulation of osteoblastic activity occurs. It has been suggested that two mechanisms operate in bone formation: the phosphatase mechanism which produces in the bone matrix fluid a condition of supersaturation with respect to bone phosphate; and an "inorganic" mechanism which favors deposition of this salt from supersaturated solutions.

TABLE SHOWING RETENTION AND ELIMINATION OF CA AND P IN RACHITIC RATS AND DURING HEALING*

(AVERAGES OF FOUR RATS, EXPRESSED ON THE BASIS OF ONE RAT PER PERIOD)

Periods	*Calcium*				*Phosphorus*			
	Intake, mg.	*Distribution (per cent of intake)*			*Intake, mg.*	*Distribution (per cent of intake)*		
		Retained	*Excreted*			*Retained*	*Excreted*	
			Urine	*Feces*			*Urine*	*Feces*
I (Rickets)	365	15.6	12.1	72.3	105	36.2	0.0	63.8
II (Healing)	163	47.2	0.7	52.1	159	39.6	20.5	39.9

* From unpublished data obtained in the senior author's laboratories. Similar observations are reported by Shohl, Bennett, and Weed: *J. Biol. Chem.*, **79**, 257 (1928).

Period I: A 5-day period in the fourth week of a high-Ca, low-P rachitogenic diet.

Period II: A 5-day period during the succeeding week, after the Ca: P ratio was diminished by substituting $CaHPO_4 \cdot 2H_2O$ for $CaCO_3$.

New light has been cast on the biochemical role of alkaline phosphatase in rickets by the work of Zetterstrom and Ljunggren,[380] who studied the action of the water-soluble phosphorylated derivative of vitamin D_2 ("D_2P"). They showed that D_2P has a remarkable capacity to activate phosphatase, especially from bone. It would appear therefore that vitamin D may function as an activator of alkaline phosphatase not only in the intestinal absorption of phosphate but also in bone deposition and in renal tubular reabsorption, all of which are involved in clinical rickets. A more general role for vitamin D in carbohydrate oxidation is suggested by *in vitro* studies[381] in which D_2P was shown to have an activating effect on the oxygen uptake of kidney mitochondria. The respiratory enzyme complex here involved is known to be concerned in the tricarboxylic acid cycle and in fatty acid oxidation.

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[380] Zetterstrom and Ljunggren: *Acta Chem. Scand.*, **5**, 283 (1951)

Efforts have been made to correlate the action of vitamin D with the important role played by the parathyroid hormone in the maintenance of calcium equilibrium in the serum. Hyperparathyroidism is accompanied by high serum calcium, and apparent dissolution of this element from the bones which, when prolonged, may lead to osteomalacia. Parathyroid extract does not cure rickets. Though there are similarities in the effects of vitamin D and parathyroid hormone on calcium mobilization, evidences of differences in their mode of action—e.g., in tetany or when given in overdosage—make doubtful a direct hormonal role in vitamin D activity.

The vitamin D requirement of man is influenced by age, color, pregnancy, lactation, the mineral content of the diet, and conditions which affect intestinal absorption. The vitamin D needs of adults and older children may be satisfied by sufficient exposure to ultraviolet radiation. According to the National Research Council, the recommended daily allowance of this vitamin is 400 units daily, except during pregnancy, lactation, and the first year of childhood, when it is increased to 800 units. See p. 1108. Vitamin D may be administered not only subcutaneously or intraperitoneally[382] but by direct absorption through the skin,[383] although the dosage requirements under these conditions are not well defined.

Even when massive doses are given, the transfer of vitamin D to human or cow's milk is limited to only a few per cent. The administration of 40,000 units per day during early lactation of mothers increased the vitamin D content from very low or zero levels to a range of 125 to 583 units per liter. Vitamin D milk of commerce is produced by direct addition of irradiated sterols or concentrates; formerly "metabolized vitamin D milk" was obtained by controlled dosage of cows.

Some success has been claimed for the more or less empirical use of large doses of vitamin D in the treatment of arthritis, but the reports in the literature are conflicting and the physiological mechanism involved is not known.

The belief that excessive doses of vitamin D were toxic owed its origin in part to unfortunate experiences with an early German product "Vigantol," which probably contained an excessive proportion of toxisterol (see p. 1252) due to overirradiation of the sterol. Later studies have shown that the margin between the therapeutic and the minimum toxic dose of vitamin D is very wide. Several hundred times ordinary therapeutic doses must be administered daily for several weeks before toxic effects are noted. It has been suggested that infants be given doses of 250,000 units twice a year for protection against rickets. The chief symptoms of toxicity due to overdosage of vitamin D are anorexia and polyuria. Calcification of soft tissues, and particularly of the renal arterioles and the aorta, are observed in advanced stages, although hypertension is not encountered. Vitamin D overdosage is indicated by a rise in serum calcium. The toxic effects disappear upon discontinuance of the dosage.

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[381] Zetterstrom: *Acta Chem. Scand.*, **5**, 343 (1951).

[382] Soames: *Biochem. J.*, **18**, 1349 (1924); Hess, Weinstock, and Helman: *J. Biol. Chem.* **63**, 305 (1925); Kramer, *et al.*: *J. Biol. Chem.*, **71**, 699 (1927).

[383] Hume, Lucas, and Smith: *Biochem. J.*, **21**, 362 (1927); Helmer and Jansen: *Studies Inst. Divi Thomae*, **1**, 83 (1937).

The calcium content of the blood serum of rachitic children is normal (9–11 mg. per 100 ml.) or only slightly lower, but the inorganic phosphorus content may be reduced to half the normal values of 4–5 mg., or even lower in severe cases. There are seasonal variations in the blood phosphate values, the minimum occurring in late winter and early spring, and the maximum in midsummer. These changes run parallel to the seasonal variation in solar ultraviolet radiation and coincide with the variations in the incidence of clinical rickets. They may also account for the spontaneous healing often observed in summer.

These observations as well as the well-known geographical distribution of rickets, have been attributed to the effect of exposure to sunlight. The therapeutic value of the sun's rays in rickets was noted by Huldschinsky, who also studied the use of artificial sources of ultraviolet radiation. Certain of these rays cause a synthesis of vitamin D from a precursor (7-dehydrocholesterol) in the skin, and a similar synthesis can be produced in animal and plant oils from this or other provitamins.

For further discussion of the clinical aspects of vitamin D, see the American Medical Association syllabus, p. 1292.

Ultraviolet Radiation. The visible region of the spectrum comprises only a small portion of the entire scale of radiant energy. The colors in the visible spectrum result from differences in the frequency—or inversely, the wavelength—of the radiations. In the invisible regions of electromagnetic vibrations, there are the longer radiations (infrared or heat rays, Hertzian, and radio waves) and the shorter radiations (ultraviolet rays, gamma rays, x-rays, etc.). The relation between these forms of radiant energy is illustrated in Fig. 287.

The antirachitic region of the spectrum extends from about 256 mμ to about 313 mμ, whereas the shortest solar radiation is about 290 mμ. It is only when the atmosphere is free from smoke, fog, dust, clouds, etc., that solar energy of wavelength shorter than 300 mμ reaches the earth, and even then its intensity is very slight. On a clear day at sea level the energy distribution is approximately 1 to 2 per cent ultraviolet, 42 to 53 per cent visible and 57 to 63 per cent infrared. The limited antirachitic protection offered from this source helps explain the frequency of rickets and the need for dietary sources of preformed vitamin D. Ultraviolet radiation exerts its maximum activating power at the wavelength of maximum absorption by the provitamin, e.g., at 281 mμ (range = 275 to 300 mμ) in the case of ergosterol. The energy required for activation is 7.5×10^{13} quanta per unit of vitamin D.

Ultraviolet energy emanates from incandescent solids. The distribution and intensity of this energy depends on the nature of the incandescent source. The most widely used sources of ultraviolet radiation in experimental or therapeutic work are the carbon arc and the quartz mercury arc. Carbon arc lamps are provided with carbons containing metallic cores of definite composition. The quartz mercury-vapor lamp consists of a quartz chamber in which an arc discharge takes place between electrodes of mercury or of mercury and tungsten. The ultraviolet component of the radiation from these lamps depends on the energy input and, in the case of the mercury vapor arc, diminishes with the age of the lamp.

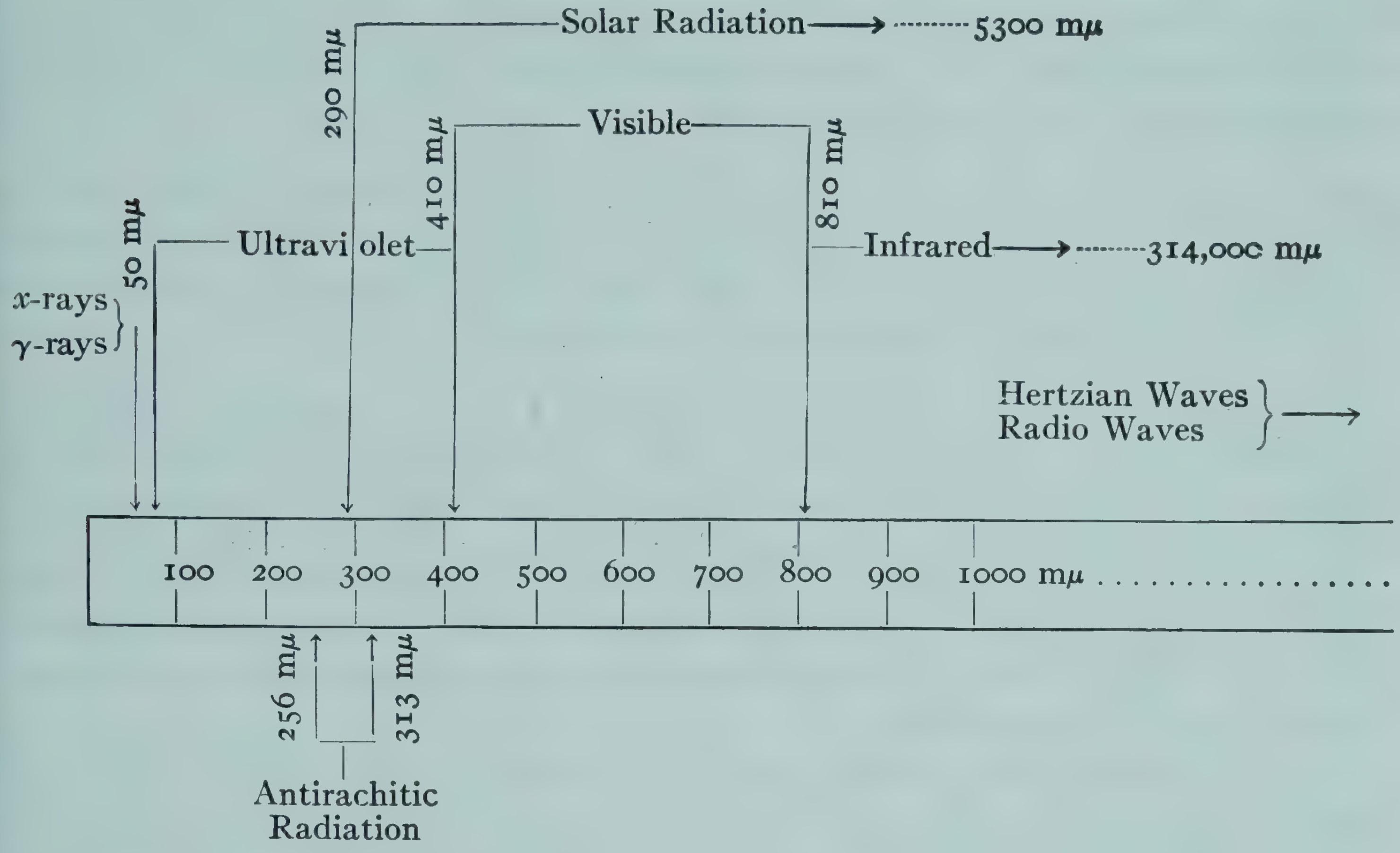

FIG. 287. CHART SHOWING DISTRIBUTION OF RADIANT ENERGY. The figures indicate the wavelengths, expressed in millimicrons.[384]

Ordinary window glass is opaque to ultraviolet radiation but quartz is transparent. Various makes of glass of special composition permit the transmission of antirachitic radiation and are used in schools, hospitals, solaria, etc. Certain plastic films are also transparent to the antirachitically active rays, and these have been used in windows of poultry houses.

The variation in distribution and intensity of ultraviolet radiation from the sun or from artificial sources makes necessary some means of measurement. No satisfactory method has been developed for analyzing the antirachitic region alone, and of the biological and photochemical methods it may be said that no two of them measure exactly the same region and none of them gives an index of the distribution of energy. The only accurate methods are those based on a physical measurement of the energy, such as those employing a thermopile and galvanometer. Radiation of any region of wavelength, selected by optical filters or a quartz monochromator, may be absorbed by a thermocouple, causing a flow of current which can be measured by a galvanometer previously standardized against radiation of known intensity. Such a method, while nonselective and reliable, is beyond the scope of most biological laboratories.

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[384] One micron (μ) = 0.001 mm.; one millimicron (mμ) = 0.001 μ; one Ångstrom unit (Å) = 0.1 mμ.

Of the photochemical methods[385] there may be mentioned (1) *Clark's*, in which the number of minutes required for a paste of lithopone, or preferably pure zinc sulfide, to darken to a definite shade is taken as the index and expressed as lithopone or ZnS units of ultraviolet energy; (2) *Pohle's*, in which the index is the time required for the ultraviolet energy to produce a blue color in a solution containing potassium iodide, sodium thiosulfate, and starch; (3) *Webster, Hill, and Eidinow's* in which the fading of the blue color in an acetone–methylene blue solution is measured by comparison with known standards; and finally (4) the method of *Anderson and Robinson*, given below.

Photochemical Method for Measuring Ultraviolet Intensity: Principle. In the presence of a uranium salt which acts as a catalyst, a solution of oxalic acid is decomposed by ultraviolet, but not by visible, radiation. The degree of decomposition is determined by titration with potassium permanganate.

Procedure. **A solution containing exactly 6.3 g. c.p. oxalic acid ($H_2C_2O_4 \cdot 2H_2O$) and 4.27 g. uranyl sulfate ($UO_2SO_4 \cdot 3H_2O$) per liter of distilled water is prepared and kept protected from the light. Place 25 ml. of this solution in a fused quartz cell 3.35 × 10 cm. in area and 1.65 cm. thick.[386] The unshielded area of the cell is exposed directly to the source of ultraviolet energy for a definite period. In the case of solar measurements a one-hour exposure is used, the angle of the cell being changed every 5 minutes to keep the rays perpendicular to the surface.**

After the exposure the contents of the cell are rinsed into a flask with 50 ml. distilled water, and 2 ml. concentrated sulfuric acid are added. The solution is then boiled and titrated while hot with a solution of potassium permanganate standardized to be equivalent to the oxalic acid solution (theoretically 3.16 g. $KMnO_4$ per liter).

CALCULATION. (25 − ml. of $KMnO_4$ used) × 0.63 = mg. oxalic acid decomposed. Duplicate determinations should agree within 0.06 mg. The maximum value found by Tonney for sunlight at Chicago was 7.12 mg. per hour. Sunburn of untanned skin (the "erythema reaction") was observed whenever the intensity exceeded half this maximum value.

Storage of Vitamin D. The animal body is able to maintain a reserve of vitamin D, the amount depending on the dietary supply and on the extent of exposure to the synthesizing influence of ultraviolet radiations. The rate of depletion of the bodily store of vitamin D, when a supply of this factor is lacking, is affected by the ratio of calcium to phosphorus in the diet, which under normal conditions should be between 1:1 and 2:1.

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[385] Clark: *Am. J. Physiol.*, **69**, 200 (1924); *Science*, **68**, 165 (1928); Pohle: *J. Am. Med. Assoc.*, **86**, 318 (1926); Webster, Hill, and Eidinow: *Lancet*, **1**, 745 (1924); Hill: *Lancet*, **2**, 299 (1924). A set of standard blue gauges, quartz tubes, and solution for use in this test is marketed by Siebe, Gorman and Co., Ltd., London, S.E. 1, England. Anderson and Robinson: *J. Am. Chem. Soc.*, **47**, 718 (1925); Tonney: *J. Prevent. Med.*, **2**, 493 (1928).

The photochemical methods have been calibrated against the radiometric method and critically reviewed by Mayerson (*Am. J. Hygiene*, **22**, 106 (1935)) with the conclusion that the oxalic acid–uranyl sulfate method is reliable and most convenient.

[386] Obtainable from the Hanovia Chemical and Manufacturing Co., Newark, N. J. The cell walls are covered with opaque paper except for a 10 sq. cm. area on the face to be exposed to the light source.

Young animals acquire some degree of antirachitic protection by virtue of the transfer of vitamin D into the mother's milk, although the efficiency of this transfer is very low (about 1 to 2 per cent). By feeding fish liver oil or irradiating the mother during the periods of gestation and lactation, the vitamin D potency of the milk can be increased somewhat, but ordinarily both cow's milk and human milk are poor sources of the antirachitic vitamin. The fortification of cow's milk with vitamin D is discussed on p. 1256.

Chemistry of Vitamin D. Five years after Huldschinsky's[387] demonstration of the therapeutic value of ultraviolet irradiation on rickets, Hess[388] and Steenbock[389] in 1924 independently revealed the discovery that antirachitic activity could be induced in foods by exposure to ultraviolet radiation. It was soon demonstrated that the antirachitic factor was contained in the nonsaponifiable fraction of activated oil, just as in the case of codliver oil. Interest then centered on the activability of phytosterol and cholesterol, the important unsaponifiable lipides of plant and animal tissue, respectively. At first it appeared that cholesterol was the specific precursor of vitamin D, but later work revealed other sterols to function in this role, viz., *ergosterol* in the lipide fraction of plants, yeasts, and fungi, and *7-dehydrocholesterol* in animal sources. Ergosterol had originally been isolated from ergot, the fungus of rye. Following the discovery of its provitamin nature it was extracted commercially from yeast. 7-Dehydrocholesterol was originally obtained from brain tissue, crustaceans, mussels, and other shellfish, but is now produced synthetically. The activated product of ergosterol is called vitamin D_2 or *calciferol*, whereas that of 7-dehydrocholesterol is vitamin D_3. From the structural formulas it will be seen that activation of these sterols involves opening of the B ring and conversion of the methyl group between the A and B rings to methylene.

The synthesis of vitamin D from ergosterol is independent of the wavelength of the ultraviolet radiation over the range of selective absorption (about 256 to 313 $m\mu$), but is a function only of the absorbed energy.[390] It is therefore possible to activate ergosterol by exposure to the unobstructed rays of the sun in spite of the fact that radiation of such short wavelength, even under ideal conditions, hardly ever exceeds 0.1 per cent of the total solar energy.[391] The efficiency of solar irradiation may be affected by a relative preponderance of inactive decomposition products produced by the longer waves.[392] Activation is also induced by means of electronic streams, x-rays, radioactive emanations, and high-frequency alternating current.

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[387] Huldschinsky: *Deut. med. Wochschr.*, **45**, 712 (1919).

[388] Hess: *Am. J. Diseases Child.*, **28**, 256 (1924); Hess and Weinstock: *J. Biol. Chem.*, **62**, 301 (1924).

[389] Steenbock and Black: *J. Biol. Chem.*, **61**, 405 (1924).

[390] Kon, Daniels, and Steenbock: *J. Am. Chem. Soc.*, **50**, 2573 (1928); Webster and Bourdillon: *Biochem. J.*, **22**, 1223 (1928); *Nature*, **123**, 244 (1929); Marshall and Knudson: *J. Am. Chem. Soc.*, **52**, 2304 (1930).

[391] Coblentz, Dorcas, and Hughes: *Bur. Standards Sci. Paper No.* 539, **21**, 535 (1926); *J. Am. Med. Assoc.*, **88**, 390 (1927); Greider and Downes: *Trans. Illum. Eng. Soc.*, **25**, 378 (1930); Forsythe and Christison: *J. Optical Soc. Am.*, **20**, 396 (1930).

[392] Lahousse and Gonnard, cited by Bills: *Physiol. Revs.*, **15**, 1 (1935).

In the ultraviolet activation of ergosterol a series of products is formed depending on the wavelength of the radiation, the duration of exposure, overheating, the nature of the solvent, the presence of oxygen, etc. The changes induced in the absorption spectrum of ergosterol by irradiation are shown in Fig. 288. The following scheme summarizes the sequence in which the isomeric products of irradiation appear:

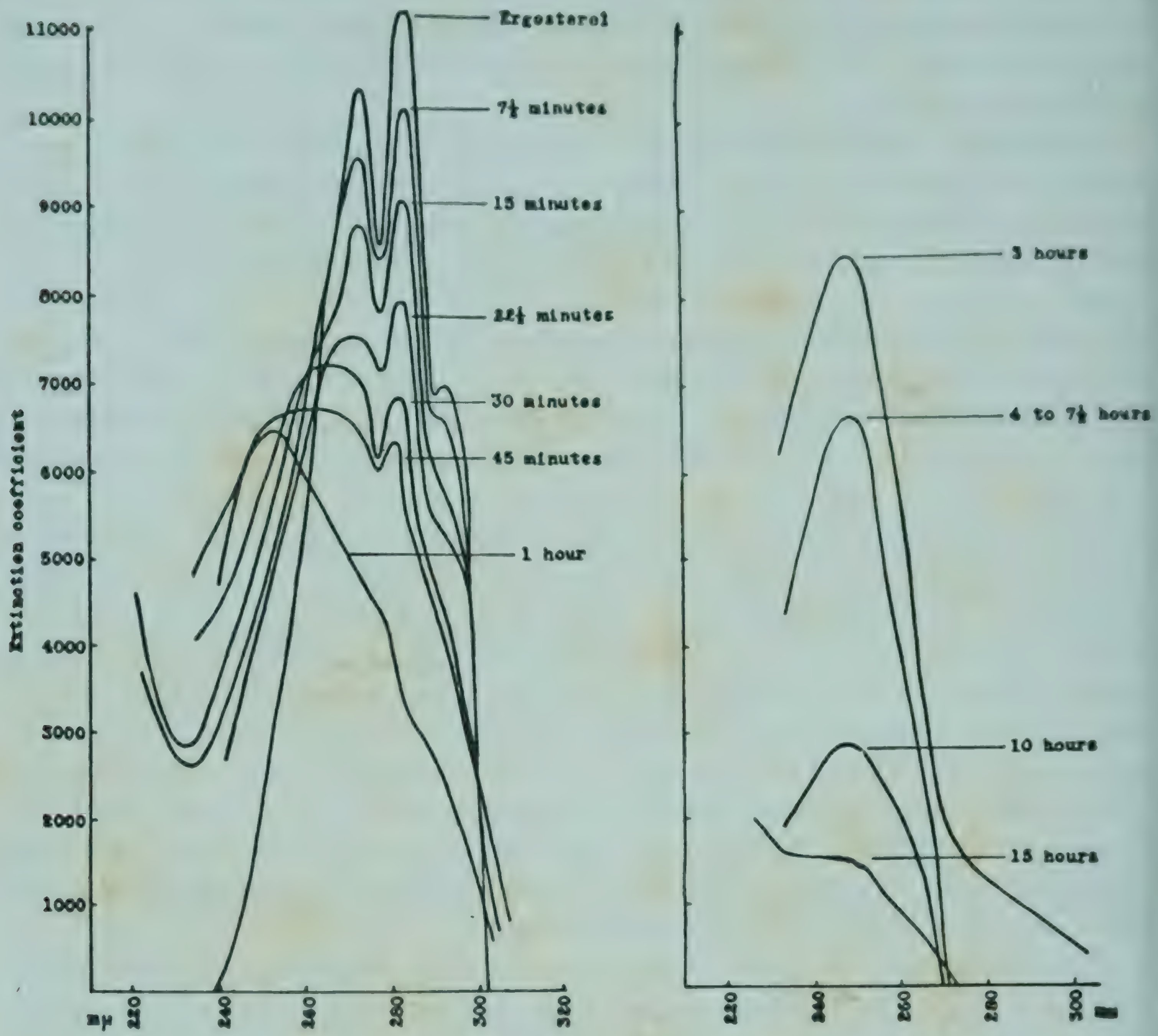
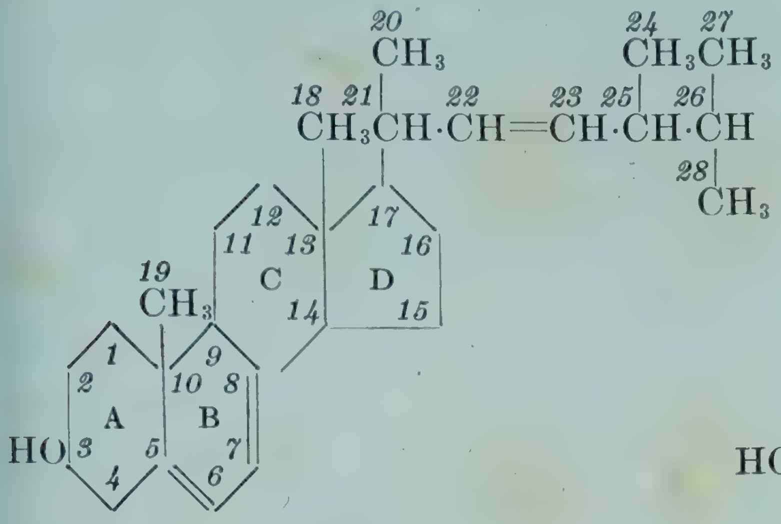


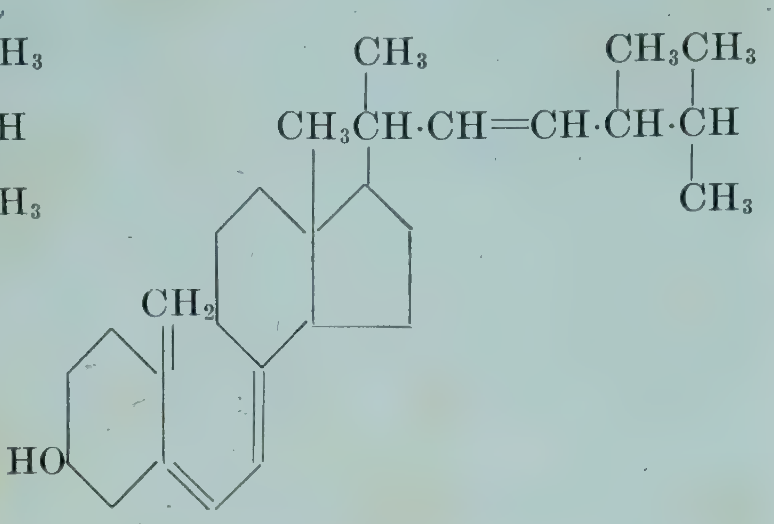
FIG. 288. EFFECT OF PROLONGED IRRADIATION ON THE ULTRAVIOLET ABSORPTION SPECTRUM OF ERGOSTEROL.
Courtesy, Bills, Honeywell, and Cox: *J. Biol. Chem.*, **80**, 557 (1928).

$$\begin{tikzcd}[column sep=small] & & & & \text{Toxisterol} \\ \underset{\textit{(Provitamin } D_2)}{\text{Ergosterol}} \arrow[r] & \text{Lumisterol} \arrow[r] & \text{Tachysterol} \arrow[r] & \underset{\textit{(Vitamin } D_2)}{\text{Calciferol}} \arrow[ur] \arrow[dr] & \\ & & & & \underset{\textit{(I and II)}}{\text{Suprasterols}} \end{tikzcd}$$

The designation vitamin D_1 was applied to what was later found to be an equimolecular addition compound of lumisterol and calciferol; calciferol itself has been called vitamin D_2. Of these irradiation products only calciferol is antirachitic. Toxisterol, as its name implies, has pronounced toxic-calcifying properties. The isomeric conversion of ergosterol to calciferol is now believed to be characterized by the opening of the B ring at C_{9-10}, resulting in a fourth unsaturated linkage, the methyl group going to methylene. A similar change occurs in the activation of 7-dehydrocholesterol (p. 1254).



Ergosterol



Calciferol

The properties of ergosterol, 7-dehydrocholesterol, and their respective activation products are shown in the following table:

	Ergosterol	*Vitamin D_2 (Calciferol)*	*7-Dehydrocholesterol*	*Vitamin D_3*
Formula..........	$C_{28}H_{43}OH$	$C_{28}H_{43}OH$	$C_{27}H_{43}OH$	$C_{27}H_{43}OH$
Double bonds.....	3	4	2	3
Melting point.....	166° C.	116° C.	143° C.	83° C.
Digitonin.........	Precipitate	No precipitate	Precipitate	No precipitate
Absorption bands.	260, 270, 282, 293.5 mμ	265 mμ	Same as ergosterol	Same as calciferol
Optical rotation...	In $CHCl_3$: $[\alpha]_D^{20} = -132°$	In C_2H_5OH: $[\alpha]_D^{20} = +102.5°$	In $CHCl_3$: $[\alpha]_D^{20} = -113.6°$	In acetone: $[\alpha]_D^{20} = +83.3°$
Vitamin D potency rats............ chicks..........	 None None	 40,000,000 u./g. 400,000 u./g.	 None None	 45,000,000 u./g. 45,000,000 u./g.

The demonstration[393] that activated ergosterol when fed to chicks on an equivalent rat-unit basis is only about one-hundredth as effective for them as codliver oil paved the way for studies proving that calciferol is not identical with naturally occurring vitamin D. By means of the comparative chick vs. rat assays, Waddell[394] showed that the provitamin in impure cholesterol was not ergosterol, since the relative species effectiveness of irradiated cholesterol is similar to that of the natural vitamin. Numerous investigations have revealed a number of sterol derivatives which can acquire antirachitic potency upon irradiation,[395] including, in addition to the natural precursors ergosterol and 7-dehydrocholesterol, 22-dihydroergosterol, 7-hydroxycholesterol, and 7-dehydrositosterol. There is also evidence for the chemical activation of cholesterol (by forming cholesterylene sulfonic acid) and of ergosterol (by treatment with nitrite).

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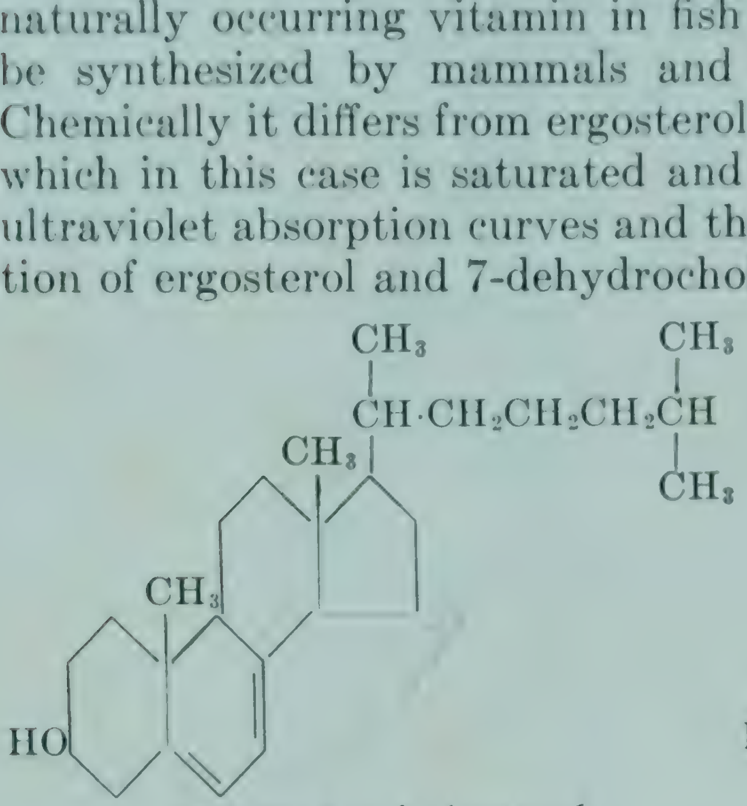
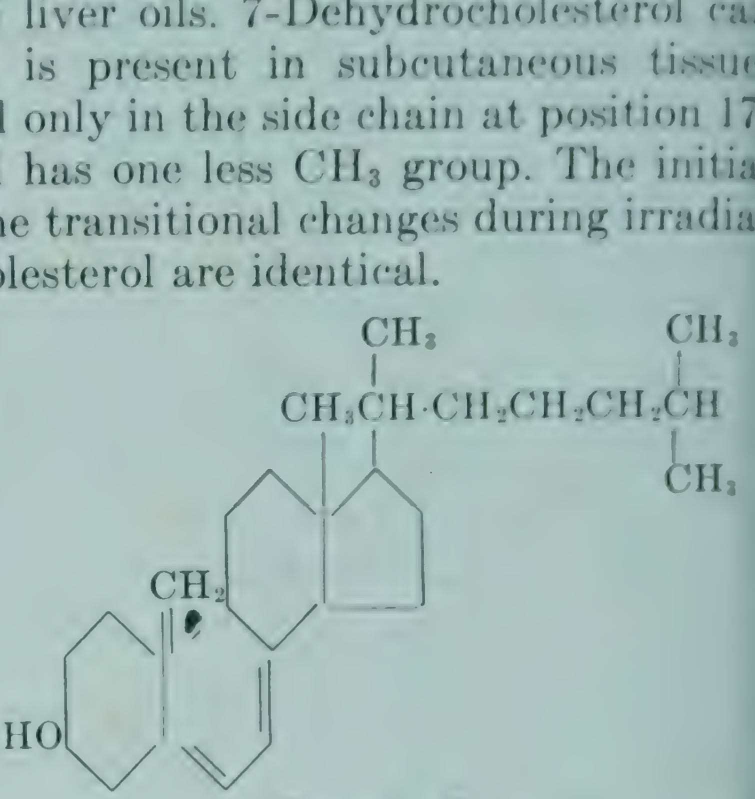
[393] Massengale and Nussmeier: *J. Biol. Chem.*, **87**, 423 (1930).
[394] Waddell: *J. Biol. Chem.*, **105**, 711 (1934).

The products of irradiation of 7-dehydrocholesterol are analogous to those of ergosterol, the antirachitic substance being identical with the naturally occurring vitamin in fish liver oils. 7-Dehydrocholesterol can be synthesized by mammals and is present in subcutaneous tissue. Chemically it differs from ergosterol only in the side chain at position 17, which in this case is saturated and has one less CH_3 group. The initial ultraviolet absorption curves and the transitional changes during irradiation of ergosterol and 7-dehydrocholesterol are identical.



7-Dehydrocholesterol



Vitamin D₃

From an extensive series of comparative rat-chick assays of the liver oils of many species of fish, Bills demonstrated the existence of more than one form of natural vitamin D. For example, the liver oil of bluefin tuna was found to be only about one-sixth as effective antirachitically for the chick as for the rat, whereas that of white sea bass was two or three times as effective, rat unit for rat unit. For the vitamin content of various fish liver oils, see the table on p. 1115.

The resistance of vitamin D to oxidation is shown by the failure of aeration of hot codliver oil or of treatment with hydrogen peroxide for 18 days to effect appreciable loss of antirachitic potency. The vitamin is not readily destroyed by gentle hydrogenation,[396] or by the action of such reducing agents as hydrogen sulfide, sulfur dioxide, or formaldehyde; on the other hand, nitrous fumes cause destruction.[397]

Vitamin D is thermostable in approximately neutral medium, as is indicated by its ability to withstand the direct steaming process used in rendering cod livers. Ninety minutes' autoclaving at 130° C. caused practically no destruction; at 140° to 145° C. the loss was slight; and at 165° to 170° C. destruction was complete.[397] In acid medium, however, gradual destruction takes place, the rate depending on the reaction and the temperature.[398] Vitamin D can withstand treatment with strong alkali even at elevated temperatures.

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[395] Bills: *Cold Spring Harbor Symp. Quant. Biol.*, **3**, 328 (1935); *J. Am. Med. Assoc.*, **108**, 13 (1937); *J. Nutrition*, **13**, 435 (1937).

[396] Dubin and Funk: *Proc. Soc. Exptl. Biol. Med.*, **21**, 139 (1923).

[397] Saiki and Fujimaki: *Bull. soc. hyg. aliment.*, **15**, 481 and 524 (1927).

[398] Bills: *J. Biol. Chem.*, **64**, 1 (1925).

Distribution of Vitamin D. The most abundant natural source of vitamin D is codliver oil. Nevertheless, whereas ordinary codliver oil possesses a vitamin D activity of approximately 100 to 200 U.S.P. units per gram, the liver oils of certain other species of fish may be many times more potent.[399] Some liver oils which are blended for medicinal use are high in vitamin D, e.g., those from various species of tuna. Other oils, e.g., those of the swordfish and halibut, are also high in vitamin A. (See table, p. 1115.) The vitamin potency of fish liver oils is affected by spawning (and hence the season of the catch and the oil content of the livers) and by the age of the fish. Varying amounts of vitamin D are also found in the body oils of many marine animals, among them salmon, herring, sardine, shrimp, oysters, etc.[400]

Whether fish are able to synthesize vitamin D, as indicated by the experiments of Bills,[399] or whether it is derived from the caplin and other forms of food they consume, has not been definitely settled. There is evidence[401] that the vitamins of codliver oil are derived indirectly from unicellular marine organisms via copepods, larval decapods, and mollusca present in marine plankton, which in turn is consumed by the cod. However, Leigh-Clare[402] failed to demonstrate the presence of vitamin D in these diatomaceous organisms, although other investigators report the presence of the vitamin in marine plankton[403] especially during the summer months when it is abundant near the surface of the water.

When the livers of cod are in the process of becoming spent, the vitamin D potency of the oil bears an inverse relation to the oil content.[404] This has been attributed to the relatively rapid depletion of the glycerides during the starvation period, leaving behind the more resistant antirachitic factor. In a large-scale experiment conducted at the Norwegian fishing grounds, however, the senior author found that at the spawning season, when the livers were rich in oil, there was no demonstrable difference in antirachitic potency between the oil of male and female cod. The livers of the females had a somewhat lower oil content but were larger than those of the males.[405]

No increase in the vitamin D content of codliver oil is observed after ultraviolet irradiation, excess exposure being destructive not only of vitamin A but of vitamin D as well.

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[399] Hess and Weinstock: *Proc. Soc. Exptl. Biol. Med.*, **23**, 407 (1926); Bills: *J. Biol. Chem.*, **72**, 751 (1927); *J. Nutrition*, **13**, 435 (1937); Notevarp: *Tids. Kjemi, Bergvesen Met.*, No. 4, 415 (1937).

[400] Nelson, *et al.: Ind. Eng. Chem.*, **22**, 1361 (1930); **23**, 1066 (1931); Truesdail and Boynton: *Ind. Eng. Chem.*, **23**, 1136 (1931); Brooks, Abernethy, and Vilbrandt: *J. Am. Chem. Soc.*, **52**, 4940 (1930); Jones, Murphy, and Nelson: *Ind. Eng. Chem.*, **20**, 205 (1928); Schmidt-Nielsen and Schmidt-Nielsen: *Chem. Abstracts*, **24**, 5798 (1930).

[401] Zilva and Drummond: *Biochem, J.*, **16**, 518 (1922). See also Jameson, Drummond, and Coward: *Biochem. J.*, **16**, 482 (1922); Copping: *Biochem. J.*, **28**, 1516 (1934); Drummond and Gunther: *J. Exptl. Biol.*, **11**, 203 (1934).

[402] Leigh-Clare: *Biochem. J.*, **21**, 368 (1927).

[403] Belloc, Fabre, and Simmonet: *Compt. rend.*, **191**, 160 (1930); Russell: *Nature*, **126**, 472 (1930).

[404] Hess, Bills, and Honeywell: *J. Am. Med. Assoc.*, **92**, 226 (1929).

[405] Hawk: Reported before the American Chemical Society, Minneapolis Meeting, 1929. Of 1779 livers examined, those from female cod (58.6 per cent of the total) averaged about 288 ml. in size and contained 37.1 per cent oil, while those from the male averaged 163 ml. and contained 43.3 per cent oil.

Vitamin D has a very limited distribution among the common foodstuffs. Of these, egg yolk probably takes first rank, although this source is quite variable, depending upon the hen's ration and upon the amount of exposure to ultraviolet radiation. The vitamin D content of butterfat is relatively low, in contrast to its vitamin A content, but is likewise somewhat higher in summer than in winter. Human milk is an even poorer source of the antirachitic vitamin than cow's milk.

Processes used for increasing the antirachitic potency of whole or evaporated cow's milk include: (*a*) feeding activated yeast or sterols to cows, a small fraction of the vitamin D being transmitted to the milk; (*b*) ultraviolet irradiation of the milk; (*c*) fortification of the milk with natural or synthetic vitamin D.[406] The last is the principal method in use in this country today. Vitamin D milk produced by direct fortification is standardized to contain 400 U.S.P. units per quart (whole milk) and provides adequate safeguard against rickets.

Antirachitic activation of foods by ultraviolet irradiation is dependent upon the presence of the provitamin. Hence certain food products—e.g., milk, egg yolk, yeast—are capable of much greater activation than others, like cornstarch, egg white, or certain cereals. Careful control is required in irradiation processes to avoid the development of rancidity or disagreeable odors, and to guard against destruction of vitamin A, riboflavin, pyridoxine, and (by overirradiation) of the vitamin D which is formed.

Plant foods and vegetable oils are notoriously deficient in vitamin D. Excellent sources of vitamin A though they are, the green leafy vegetables lack the antirachitic factor in significant amounts even when grown in the summer months. Sun-drying of hay or alfalfa results in a considerable increase in antirachitic potency. Some activation occurs during fermentation of cocoa, owing to solar radiation of the sterols present in contaminating yeasts or fungi.

Certain cereals, notably oatmeal, contain a factor which actively inhibits calcification.[407] Not only is part of their phosphorus content nonassimilable, but it occurs in the form of inositol hexaphosphoric acid or phytin[408] which binds calcium and iron and thus interferes with their absorption.

DETERMINATION OF VITAMIN D

CHEMICAL METHODS

The determination of vitamin D by physical and chemical methods is complicated by the multiplicity of forms in which it may occur and by the interference of inactive sterols and of vitamin A in the color reactions. By chromatographing the unsaponifiable extract and measuring the spectrophotometric absorption of the eluate at 265 mμ, chemical assay of irradiated sterols or similar high-potency materials is possible.[409] Similar methods employing modified antimony trichloride reagents have been used for the assay of fish liver oils, but with less success owing to their lower vitamin D potency and the interference of vitamin A. The antimony trichloride reaction differentiates between ergosterol and 7-dehydrocholesterol, which are indistinguishable spectrophotometrically.[410]

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[406] "The Present Status of Vitamin D Milk," Rept. of Council on Foods: *J. Am. Med. Assoc.*, **108**, 206 (1937). See also Jeans: *J. Am. Med. Assoc.*, **106**, 2066 (1936).

[407] Mellanby: *Brit. Med., J.*, **2**, 849 (1922); Green and Mellanby: *Biochem. J.*, **22**, 102 (1928).

[408] Bruce and Callow: *Biochem. J.*, **28**, 512 and 517 (1934); McCance and Widdowson: *Biochem. J.*, **29**, 2694 (1935); Lowe and Steenbock: *Biochem. J.*, **30**, 1126 and 1991 (1936).

Green has developed a promising chemical procedure for vitamin D in fish liver oils[411] involving a series of steps designed to eliminate the interference by sterols and vitamin A with color reactions. The unsaponifiable extract is chromatographed on acid-activated floridin to destroy vitamin A; the carbon tetrachloride eluate is evaporated to dryness and taken up in 72 per cent ethanol to precipitate interfering provitamins and sterols; the filtrate is dried and taken up in chloroform, and the vitamin D ("apparent calciferol") is estimated spectrophotometrically after reacting with the antimony trichloride–acetyl chloride reagent of Nield, Russell, and Zimmerli.[412] While the method is claimed to give good agreement with bioassays, the "detailed steps necessary for the analysis of vitamin A-containing oil have generally to be decided for each particular sample" on the basis of the ratio of vitamin A to vitamin D and the percentage of unsaponifiable matter. By means of an iodine trichloride reaction the method has been adapted for the partial analysis of the irradiation products of ergosterol and 7-dehydrocholesterol, some of which remain unidentified.[413]

BIOLOGICAL METHODS

The quantitative study of vitamin D activity is based on the determination of the comparative amounts of test materials and of standard vitamin D preparations necessary to induce healing of rickets in rats or to maintain normal calcification in chicks. The production of experimental rickets of a moderate but uniform degree of severity within a definite period of time is the primary requisite for reproducibility of results. Various methods differ in respect to the composition of the rachitogenic diet, the criteria for the establishment of the presence and degree of rickets, and the basis for judging the degree of healing. The rat curative assay (U.S.P.) is employed for the evaluation of the vitamin in foods or pharmaceutical products intended for human (or other mammalian) use; because of the relative insensitivity of fowl to vitamin D_2, the chick method (A.O.A.C.) is employed in the assay of vitamin D intended for feeding poultry.

Ordinarily diets used for the production of experimental vitamin-deficiency diseases are characterized by being complete in all respects (i.e., as regards proteins, calories, minerals, and vitamins) except for the absence of the vitamin in question. This, however, is not so in the case of the vitamin D assay with rats because decalcification does not occur readily in the absence of vitamin D unless the normal calcium:phosphorus ratio is disturbed. The analog of clinical rickets may be produced experimentally in rats by feeding a vitamin-D-free diet possessing a Ca:P ratio of about 4.5:1 instead of the normal ratio ranging from 1:1 to 2:1.

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[409] Ewing, Kingsley, Brown, and Emmett: *Ind. Eng. Chem., Anal. Ed.*, **15**, 301 (1943); Ewing, Powell, Brown, and Emmett: *Anal. Chem.*, **20**, 317 (1948).

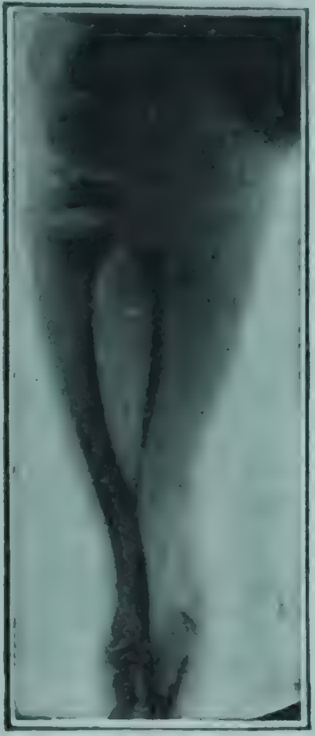
[410] Lamb, Müller, and Beach: *Ind. Eng. Chem., Anal. Ed.*, **18**, 187 (1946).

[411] Green: *Biochem. J.*, **49**, 243 (1951).

[412] Nield, Russell, and Zimmerli: *J. Biol. Chem.*, **136**, 73 (1940).

To prepare this reagent dissolve 18 g. antimony trichloride in 100 ml. purified chloroform, filter, and add 2 ml. redistilled acetyl chloride. Prepare fresh daily.

[413] Green: *Biochem. J.*, **49**, 232 (1951).



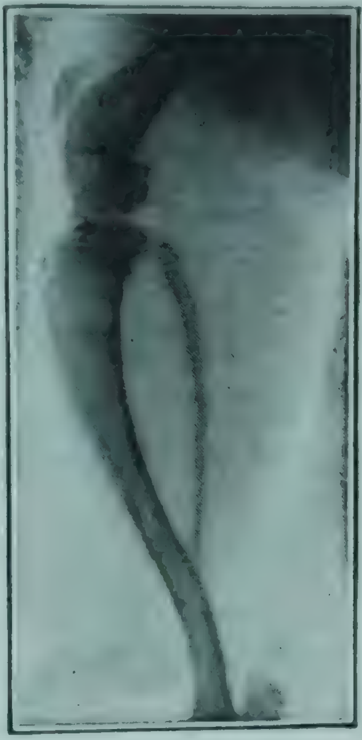


FIG. 289. (*Left*) ACTIVE RICKETS.

Roentgenograph of knee joint of rat on Steenbock rachitogenic diet No. 2965 for 17 days. Note especially broad bands of uncalcified cartilage near ends of tibia and femur.

FIG. 290. (*Right*) HEALED RICKETS.

Roentgenograph of knee joint shown in Fig. 289 after the rat had been fed a codliver-oil concentrate 14 days.

After 18 to 21 days on a standard rachitogenic diet, the animals develop a decalcified zone in the epiphyseal region of the long bones (the so-called "rachitic metaphysis") which may be readily demonstrated roentgenographically (see Fig. 289). At this time gross symptoms of rickets, such as shambling gait or enlargement of the knee joint, are not yet apparent. Hence it is desirable in order to establish the presence of the rachitic lesions that the animals be x-rayed or fluoroscoped or that some of them be sacrificed and their leg bones examined histologically.

After three weeks the bone ash values, expressed on a dry fat-free basis, drop from 35–45 per cent, the value at the time the rats are placed on the rachitogenic diet, to 25–35 per cent. At this age the normal ash content should be about 50–60 per cent. Similarly, the inorganic phosphorus content of the blood decreases from the normal level (for rats) of 8–10 mg. per 100 ml. of serum, to about 2–3 mg.[414] During this period the animals continue to grow, but at a somewhat subnormal rate, the more rapidly growing animals exhibiting rickets of greater severity.

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[414] Bethke, Nelson, and Steenbock: *J. Biol. Chem.*, **58**, 71 (1923); Dutcher, Creighton, and Rothrock: *J. Biol. Chem.*, **66**, 401 (1925); Hammett: *J. Biol. Chem.*, **64**, 409 (1925).

Following the preparatory period, the animals are fed the same diet plus the material to be assayed, which may be incorporated in the diet or preferably fed as a supplement. Negative controls are allowed to continue on the basal diet without antirachitic supplements, while positive controls receive a daily dose of a standard source of vitamin D, viz., the International or U.S. Pharmacopeia Vitamin D Reference Oil. It is important that food materials fed as supplements do not appreciably alter the Ca:P ratio of the diet. In the event of this possibility the assay should be controlled by including the ash of the test material in the diet of the negative controls, or preferably feeding the supplement in the form of its ether extract.

Rats which lose weight or fail to consume sufficient food must be discarded, because starvation is attended by a remobilization of tissue phosphate and spontaneous healing of rickets. The animals must be shielded against exposure to sunlight or even against undue exposure to filtered daylight.

The duration of the test period depends upon the criterion employed for healing. In the roentgenographic method, a six-day curative period may be used.[415] (See Figs. 289 and 290.) This method has the advantage of permitting each animal to serve as its own control, and may be conveniently combined with the histological technique as prescribed by the U.S. Pharmacopeia. The examination of stained sections of the leg bones (Line Test, see Fig. 291) is conducted at the end of the assay period.

The feces of rats on a rachitogenic diet tend to become alkaline, but return to the normal slightly acid condition during the process of healing.[416] The increase in pH is localized in the cecum and colon rather than in the small intestine, which is the main site of calcium and phosphorus absorption. A concomitant shift of urinary pH occurs in the opposite direction. These pH changes in rickets are reversible with vitamin D therapy.[417] Efforts to adapt fecal pH shift to the quantitative estimation of vitamin D[418] have failed owing to the small (about one pH unit) and variable difference and the interfering effect of extraneous factors.

Fish liver oils and vitamin D concentrates are important constituents of poultry rations. Since the rat assay is an inadequate measure of the vitamin D value of certain fish oils and sterols for avian species, a quantitative method of vitamin D assay employing chicks has been adopted by the Association of Official Agricultural Chemists.[419] This is a preventive technique based on a comparison of the average bone-ash content of the assay group with that of reference groups receiving graded doses of the Standard. *One A.O.A.C. chick unit* of vitamin D is equal in biological activity for the chick to one unit of vitamin D in the U.S.P. Reference Standard.

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[415] Poulsson and Lövenskiold: *Biochem. J.*, **22**, 135 (1928).

[416] Zucker and Matzner: *Proc. Soc. Exptl. Biol. Med.*, **21**, 186 (1924).

[417] Steenbock, Bellin, and Wiest: *J. Biol. Chem.*, **193**, 843 (1951).

[418] Jephcott and Bacharach: *Biochem. J.*, **20**, 1351 (1926); **22**, 60 (1928); *J. Biol. Chem.*, **82**, 751 (1929); Shohl and Bing: *J. Biol. Chem.*, **79**, 269 (1928); Oser: *J. Biol. Chem.*, **80**, 487 (1928); Coward: *Quart. J. Pharm.*, **1**, 27 (1928).

[419] *J. Assoc. Official Agr. Chem.*, **20**, 72 (1937). Extensive studies of chick assay methods have been reported by Massengale and Bills: *J. Nutrition*, **12**, 429 (1936) and by Halvorson and Lachat: *J. Assoc. Official Agr. Chem.*, **19**, 628, 637 and 647 (1936); Lachat: *Ibid.*, **20**, 450 (1937); Griem: *Ibid.*, **20**, 438 (1937).

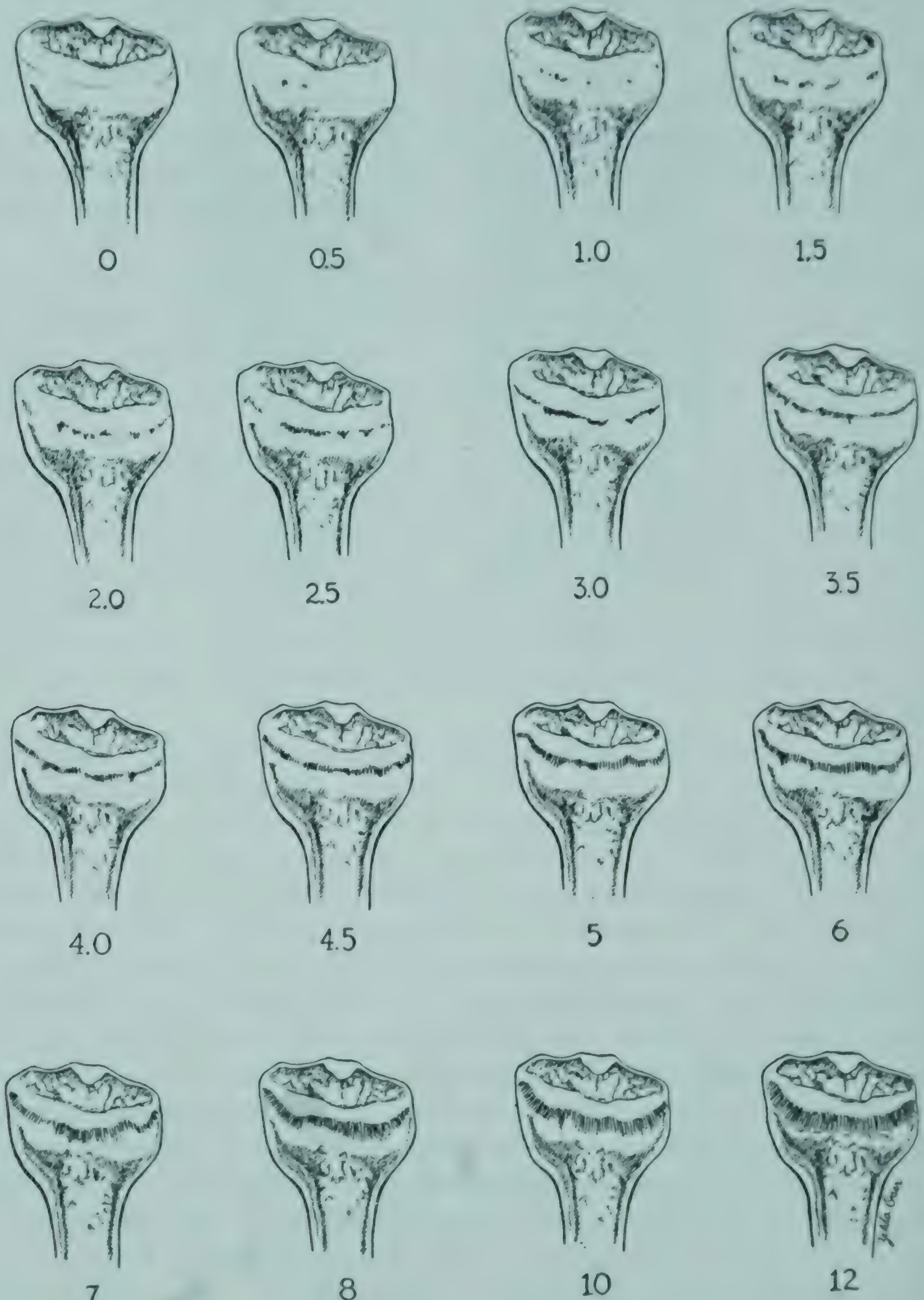

Fig. 291. Chart for Interpretation of Line Test of Tibia.

Based on 7-day line test responses according to the U.S. Pharmacopeia procedure. Note that on this scale of numerical ratings 0.5 corresponds to minimal recalcification ("positive macroscopic evidence of calcification"); 4.0 corresponds to a narrow continuous "line" of calcification along the metaphyseal junction; and 12 falls somewhat short of complete healing, but is the maximal response in a 7-day test.

ASSAYS FOR VITAMINS A AND D

Biological Methods of the U.S. Pharmacopeia XIV.[420]

DEFINITIONS. As used herein, unless the context otherwise indicates, the term *assayer* means the individual immediately responsible for the interpretation of the assay; the term *assay group* means the group of rats to which the assay oil shall be administered during the assay period; the term *assay oil* means the oil under examination for its vitamin potency; the term *assay period for the vitamin A assay* means the interval in the life of a rat between the last day of the depletion period and the twenty-ninth day thereafter or between the last day of the depletion period and the death of the rat; the term *assay period for the vitamin D assay* means the interval in the life of a rat between the last day of the depletion period and the eighth day thereafter; the term *assemble* means the procedure by which rats are selected and assigned to groups for the purpose of feeding, care, and observation; the term *control group* means the group of rats receiving no assay oil during the assay period; the term *declining weight* means the condition of a rat when its body weight on any given day is equal to or less than the body weight of the rat on the seventh day prior to the given day;[421] the term *depletion period* means the interval in the life of a rat between the last day of the preliminary period and the first day of the assay period; the term *dose* means the quantity of the U.S.P. Vitamin A Reference Standard or of the assay oil to be fed at specified intervals to a rat during the assay period; the term *fed* means made readily available to the rat or administered to the rat by mouth; the term *ground gluten* means the clean, sound product made from wheat flour by the almost complete removal of starch, and contains not more than 10 per cent of moisture, and, calculated on the water-free basis, not less than 14.2 per cent of nitrogen, not less than 15 per cent of nitrogen-free extract (using the protein factor 5.7), and not more than 5.5 per cent of starch (as determined by the diastase method[422]); the term *group for the vitamin A assay* (biological) means six or more rats maintained on the same required dietary regimen during the assay period; the term *group for the vitamin D assay* means seven or more rats maintained on the same required dietary regimen during the assay period; the term *ophthalmia* means a pathological state of the eye and/or the conjunctiva and/or the tissues anatomically related to the eye, readily discernible macroscopically and usually associated with vitamin A deficiency; the term *preliminary period* means the interval in the life of a rat between the seventh day after birth and the first day of the depletion period; the term *rachitogenic diet* means a uniform mixture of the food materials, and in the proportions named, in either of the following formulas:

Rachitogenic Diet No. 1	
Whole Yellow Maize, ground	33 per cent
Whole Wheat, ground	33 per cent
Ground Gluten	15 per cent
Gelatin	15 per cent
Calcium Carbonate (U.S.P.)	3 per cent
Sodium Chloride (U.S.P.)	1 per cent

Rachitogenic Diet No. 2	
Whole Yellow Maize, ground	76 per cent
Ground Gluten	20 per cent
Calcium Carbonate (U.S.P.)	3 per cent
Sodium Chloride (U.S.P.)	1 per cent

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[420] Grateful acknowledgment for permission to reproduce these methods is made to Dr. Lloyd C. Miller and the Board of Trustees of the U.S. Pharmacopeial Convention, Inc.

[421] In this connection it is well to remember that rats manifest a diurnal variation in body weight averaging about 2 g. Because of the severity of avitaminosis and the high mortality that often results when rats remain too long on the depletion diet, many workers prefer to reduce the period of "declining weight" to four or five days. Differences in pathological symptoms which arise at the end of the depletion period are responsible for much of the variation encountered in biological assays for vitamin A. It has therefore been suggested that preventive, rather than curative, technique might yield more uniform results.

[422] Association of Official Agricultural Chemists: *Official and Tentative Methods of Analysis*, 6th ed., 1945, p. 410, method II; p. 577, method 34.53.

The term *vitamin A test diet* means a food material consisting of the following proportions of the named ingredients of the quality specified:

Vitamin A Test Diet

Casein	18 per cent
Salt Mixture (see below)	4 per cent
Yeast, dried (U.S.P.)	8 per cent
Starch	65 per cent
Cottonseed Oil (U.S.P.)	5 per cent
Vitamin D, a sufficient amount	

No less than 3 U.S.P. Units of vitamin D shall be provided in each gram of diet and this vitamin shall be carried by the yeast or the cottonseed oil. The ingredients of the vitamin A test diet shall be free from vitamin A or shall have been treated so as to reduce the vitamin A content to such a degree that when the vitamin A test diet is fed to the control group two-thirds or more of the rats shall manifest, prior to the eleventh day of the assay period, symptoms of vitamin A deficiency characterized by both declining weight and ophthalmia.

Salt Mixture

Calcium Carbonate (U.S.P.)		68.6 g.
Calcium Citrate (U.S.P. Reagent)		308.3 g.
Calcium Biphosphate (U.S.P. Reagent)		112.8 g.
Magnesium Carbonate (U.S.P.)		35.2 g.
Magnesium Sulfate, Anhydrous (U.S.P. Reagent)		38.3 g.
Potassium Chloride (U.S.P.)		124.7 g.
Dibasic Potassium Phosphate (U.S.P. Reagent)		218.8 g.
Sodium Chloride (U.S.P.)		77.1 g.
Cupric Sulfate (U.S.P.)	0.48 g.	16.2 g.
Ferric Ammonium Citrate (U.S.P.)	96.71 g.	
Manganese Sulfate (U.S.P. Reagent)	1.24 g.	
Ammonium Alum (U.S.P. Reagent)	0.57 g.	
Potassium Iodide (U.S.P.)	0.25 g.	
Sodium Fluoride (U.S.P. Reagent)	0.75 g.	
To make	100.00 g.	
To make		1000.0 g.

Mix the finely powdered salts uniformly.

The biological assay of an oil for vitamin A and vitamin D potency shall be by comparison with the U.S.P. Vitamin A Reference Standard[423] and U.S.P. Vitamin D Reference Standard, respectively, by assay procedures conforming in all respects to the following specifications:

Assay for Vitamin A—Biological Method

For products for which the spectrophotometric method of vitamin A assay is not applicable use the biological method which follows:

The Vitamin A biological assay, comprising the recording of observations of groups of rats throughout specified periods of their lives, while being maintained on specified dietary regimens, and the interpretation of such data, is as follows:

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[423] The U.S.P. Vitamin A Reference Standard consists of an encapsulated solution of crystalline vitamin A acetate in cottonseed oil.

This oil solution is assigned a value of 10,000 U.S.P. vitamin A units per gram. The content of each capsule is assigned a value of 2500 U.S.P. vitamin A units.

PRELIMINARY PERIOD. Throughout the preliminary period each rat shall be raised under the immediate supervision of, or according to directions specified by, the assayer. Throughout the preliminary period the rats shall be maintained on a dietary regimen which shall provide for normal development in all respects, except that the supply of vitamin A, or precursors of vitamin A, shall be limited to such a degree that rats weighing between 40 and 50 g. and not exceeding 28 days of age and subsisting on a suitable vitamin A deficient ration and water for an interval not exceeding 45 days shall manifest symptoms characteristic of vitamin A deficiency.

DEPLETION PERIOD. A rat shall be suitable for the depletion period when the age of the rat does not exceed 28 days, and if the body weight of the rat shall exceed 39 g., and does not exceed 50 g., and if the animal manifests no evidence of injury, or disease, or anatomical abnormality which might hinder growth and development. Throughout the depletion period each rat shall be provided with the vitamin A test diet and water (U.S.P.), ad libitum, and during this period no other dietary supplement shall be available to the animal.

ASSEMBLING RATS INTO GROUPS FOR THE ASSAY PERIOD. Rats which are suitable for the assay period shall be assembled into groups. For each assay oil there shall be one or more assay groups. In the assay of one assay oil there shall be provided at least one control group and at least one reference group, but one control group and one reference group may be used for the concurrent assay of more than one assay oil. The interval of assembling rats into groups shall not exceed 60 days but the depletion period of rats in any one litter shall not vary by more than 14 days. On any one day during the interval of assembling rats into groups, the total number of rats that shall have been assigned to make up any one group shall not exceed by more than two the number of rats that shall have been assigned to make up any other group. When the assembling of all groups shall have been completed, the total number of rats in each group shall be the same, and the number of rats of one sex in each group shall be the same. Not more than three rats from one litter shall be assigned to one group. When the assembling of all groups shall have been completed, the average weight of the rats in any one group on the day beginning the assay period shall not exceed by more than 10 g. the average weight of the rats in any other group on the day beginning the assay period.

ASSAY PERIOD. A rat shall be suitable for the assay period, provided that the depletion period shall have exceeded 18 days and shall not have exceeded 45 days, and provided that a rat shall manifest evidence of vitamin A deficiency characterized by declining weight and/or ophthalmia. Throughout the assay period each rat of the control, reference, and assay groups shall be kept in an individual cage and shall be provided with the vitamin A test diet and water (U.S.P.), ad libitum. Throughout the assay period each rat in any assay group shall be fed two doses each week of the assay oil, and throughout the assay period each rat in any one reference group shall be fed two doses each week of the U.S.P. Vitamin A Reference Standard. The first dose of the assay oil or of the U.S.P. Vitamin A Reference Standard shall be fed on the day beginning the assay period, and succeeding doses shall be fed at intervals of not less than 3 nor more than 4 days. The U.S.P. Vitamin A Reference Standard and/or the assay oil may be diluted before feeding with cottonseed oil (U.S.P.). Diluted oil shall be stored in the dark at a temperature not exceeding 10° C. The period of storage shall not exceed 7 days. Not more than 0.2 ml. of the diluted oil shall be fed in a single dose. During the assay period all conditions of environment shall be maintained as uniformly as possible with respect to the assay, reference, and control groups.

RECORDING OF DATA. On the day beginning the depletion period and at intervals of not more than 7 days for the first 21 days of that period, there shall be a record made of the body weight of each rat. From the twenty-first day of the depletion period until the end of the assay period a record shall be made of the body weight and eye condition of each rat at intervals not exceeding 5 days. The eye condition shall be designated as normal, watery, sensitive to light, swollen, bloody exudate, purulent, opacity of cornea, or any combination of these terms. A record shall be made of the failure of a rat to consume the prescribed daily dose of Reference Standard or assay oil.

VITAMIN A POTENCY OF THE ASSAY OIL. In determining the vitamin A potency of the assay oil, the performance of the rats of the assay and reference groups shall be calculated for each group on the basis of the difference between the average weight of the surviving rats and the average weight of the same rats on the day beginning the assay period. The data from the reference group shall be considered valid for establishing the vitamin A potency of the assay oil only when two-thirds or more of the total number of animals comprising a reference group shall have made individually between the beginning day of the assay period and the twenty-eighth day thereafter an increase in body weight which shall equal or exceed 12 g. and shall not exceed 60 g., and the data from an assay or reference group shall be considered valid for establishing the vitamin A potency of the assay oil only when two-thirds or more, but not less than 6, of the rats of an assay or reference group have survived 28 days of the assay period. The data from an assay group shall be considered valid for establishing the vitamin A potency of an assay oil only when two-thirds or more, but not less than 6 rats, shall have made individually between the beginning day of the assay period and the twenty-eighth day thereafter an increase in body weight which shall equal or exceed 12 g. The data from a rat shall be considered valid for establishing the average performance of a reference or assay group only on the condition that the rat has consumed the prescribed doses during the assay period. A vitamin A biological assay shall not be considered valid unless two-thirds or more of the total number of animals comprising the control group shall, prior to the eleventh day of the assay period, manifest symptoms of vitamin A deficiency characterized by both declining weight and ophthalmia.

CALCULATION OF VITAMIN A POTENCY

Let R equal the total quantity of U.S.P. Vitamin A Reference Standard in milligrams fed to each rat in a reference group and necessary to produce in a reference group an average gain in weight, G, of not less than 12 g. nor more than 60 g.

Let A equal the total quantity of assay oil in milligrams fed to each rat in an assay group and necessary to produce in an assay group an average gain in weight equal to or greater than G.

MINIMUM STANDARD. If the product of $\left(\frac{R}{A}\right) \times$ (units per g. of vitamin A contained in the U.S.P. Vitamin A Reference Standard) is equal to or greater than the minimum standard in U.S.P. units per g. for the oil assayed, then the assay oil meets the minimum standard for vitamin A potency.

MAXIMUM STANDARD. If the product of $\left(\frac{R}{A}\right) \times$ (units per g. of vitamin A contained in the U.S.P. Vitamin A Reference Standard) is equal to or less than the maximum standard in U.S.P. units per g. for the oil assayed, then the assay oil meets the maximum standard for vitamin A potency.

ASSAY FOR VITAMIN D—BIOLOGICAL METHOD

The vitamin D biological assay, comprising the recording of observations of groups of rats, throughout specified periods of their lives, while being maintained on specified dietary regimens, and the interpretation of such data, is as follows:

PRELIMINARY PERIOD. Throughout the preliminary period each rat shall be raised under the immediate supervision of, or according to directions specified by, the assayer. Throughout the preliminary period the rats shall be maintained on a dietary regimen which shall provide for normal development in all respects, except that the supply of vitamin D shall be limited to such a degree that rats, weighing between 40 and 60 g. at an age of 21 to 30 days, and subsisting for an interval of 3 weeks on a suitable rachitogenic diet, shall manifest evidence of severe rickets.

DEPLETION PERIOD. A rat shall be suitable for the depletion period when the age of the rat does not exceed 30 days, and if the body weight of the rat shall exceed 44 g., and does not exceed 60 g., and if the animal manifests no evidence of injury, or disease, or anatomical abnormality which might hinder growth and development. Throughout the depletion period each rat shall be provided with the rachitogenic diet and water (U.S.P.), ad libitum, and during this period no other dietary supplement shall be available to the animal.

ASSEMBLING RATS INTO GROUPS FOR THE ASSAY PERIOD. Rats which are suitable for the assay period shall be assembled into groups. For each assay oil there shall be one or more assay groups. In the assay of one assay oil there shall be provided at least one reference group, but one reference group may be used for the concurrent assay of more than one assay oil. The interval of assembling rats into groups shall not exceed 60 days. On any one day during the interval of assembling rats into groups, the total number of rats that shall have been assigned to make up any one group shall not exceed by more than two the number of rats that shall have been assigned to make up any other group. When the assembling of all groups shall have been completed, the total number of rats in each group shall be the same. Not more than three rats from one litter shall be assigned to the assay group unless an equal number of rats from the same litter are assigned to the reference group. When the assembling of all groups shall have been completed, the average weight of the rats in any one group on the day beginning the assay period shall not exceed by more than 8 g. the average weight of the rats in any other group on the day beginning the assay period.

ASSAY PERIOD. A rat shall be suitable for the assay period, provided that the depletion period shall have exceeded 18 days and shall not have exceeded 25 days, and provided that a rat shall manifest evidence of rickets characterized by a distinctive, wabbly, rachitic gait and by enlarged joints. The presence of rickets may also be established by examination of a leg bone of one member of a litter by the "line test" described below. Each rat shall be kept in an individual cage and shall be provided with the rachitogenic diet and water (U.S.P.), ad libitum. On any calendar day of the assay period, the assay and reference groups shall receive a rachitogenic diet compounded from the same lots of ingredients. On the first and fourth calendar days of the assay period each rat in any one assay group shall be fed one-half the total dose of the assay oil, and on the first and fourth calendar days of the assay period each rat in any one reference group shall be fed one-half the total dose of the U.S.P. Vitamin D Reference Standard, except that when the fourth calendar day of an assay period is a holiday, the dose of the assay oil or the U.S.P. Vitamin D Reference Standard may be fed on the preceding day. At the termination of the assay period, each rat shall be killed and one or more leg bones examined for healing of the rachitic metaphysis according to the "line test" described below. The reference oil and/or the assay oil may be diluted before feeding with cottonseed oil (U.S.P.). The diluted oil shall be stored in the dark at a temperature not exceeding 10° C., the storage period not to exceed 30 days. Not more than 0.2 ml. of the diluted oil shall be fed in a single dose. During the assay period all conditions of environment (particularly with reference to physiologically active radiations) shall be maintained as uniformly as possible with respect to the assay and reference groups.

LINE TEST. The line test shall be made on the distal end of the radius.[424] The end of the bone is removed from the animal and cleaned of adhering tissue. A longitudinal median section shall be made through the end of the bone with a clean, sharp blade to expose a plane surface through the junction of the epiphysis and diaphysis. Both sections of the bone shall be rinsed in distilled water and shall then immediately be immersed in a 2 per cent aqueous solution of silver nitrate for 1 minute. The sections shall then be rinsed in distilled water and the sectioned surfaces of the bone shall be exposed in water to daylight or other source of actinic light until the calcified areas have developed a clearly defined stain without marked discoloration of the uncalcified areas.

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[424] Many assayers prefer the use of the tibia and it is probable that this will be optional in the next revision of the U.S.P. assay for vitamin D. A chart showing the healing responses in the tibia is shown in Fig. 291, although the U.S. Pharmacopeia XIV prescribes and illustrates the responses in the radius. More significant than the particular bone used in the line test is the arbitrary scoring scale which should yield a linear dose: response curve.

Records shall be made immediately of the extent and degree of calcification of the rachitic metaphysis of every section. It shall be permissible to use modifications of the described procedure for staining, provided that such modified procedures clearly differentiate between calcified and uncalcified areas.

RECORDING OF DATA. On the day beginning the assay period and on the seventh day thereafter, a record shall be made of the body weight of each rat. A record shall be made of the quantity of rachitogenic diet consumed per rat during the assay period. Numerical values shall be assigned to the extent and degree of calcification of the rachitic metaphysis of the bones examined by the line test so that it will be possible to average the performance of each group. A Line Test Chart, that may be used for this purpose, is presented below.[425]

VITAMIN D POTENCY OF THE ASSAY OIL. In determining the vitamin D potency of the assay oil, the average performance of groups with respect to healing of the rachitic metaphysis shall be considered, provided that the average performance of a reference group with respect to calcification of the rachitic metaphysis shall be determined by the data from rats which individually show an extent and degree of calcification in the rachitic metaphysis as determined by the line test equal to or greater than a condition described as a positive macroscopic evidence of calcification, but no greater than an extent and degree of calcification represented by the value 8 in the Line Test Chart[425] presented below. The data from a reference group shall be considered valid for establishing the vitamin D potency of the assay oil only when two-thirds or more, but not less than seven rats, show individually an extent and degree of calcification of the rachitic metaphysis equal to or greater than a condition described as positive macroscopic evidence of calcification, but no greater than an extent and degree of calcification represented by the value 8 in the Line Test Chart[425] presented below. The data from an assay group shall be considered valid for establishing the vitamin D potency of an assay oil only when two-thirds or more, but not less than seven rats, show individually an extent and degree of calcification of the rachitic metaphysis equal to or greater than a condition described as positive macroscopic evidence of calcification. The data from a rat shall be considered valid for establishing the average performance of a group only on the condition that the weight of the rat at the termination of the assay period shall equal or exceed the weight of the rat on the beginning day of the assay period and that the rat has consumed 28 g. or more of the rachitogenic diet during the assay period and on the condition that the rat has consumed each prescribed dose of assay oil within 24 hours from the time it was fed.

CALCULATION OF VITAMIN D POTENCY

Let R equal the total quantity of U.S.P. Vitamin D Reference Standard in milligrams fed to each rat in a reference group and necessary to produce in a reference group an average extent and degree of calcification, C, not less than a condition described as positive macroscopic evidence of calcification but less than the degree of calcification represented by the value 8 in the Line Test Chart.

Let A equal the total quantity of assay oil in milligrams fed to each rat in an assay group and necessary to produce in an assay group an average extent and degree of calcification equal to or greater than C.

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[425] This chart of the line test responses as seen in the radius has been omitted. See footnote 424, p. 1265.

MINIMUM STANDARD. If the product of $\left(\frac{R}{A}\right)$ × (units per g. of vitamin D contained in the U.S.P. Vitamin D Reference Standard) is equal to or greater than the minimum standard in U.S.P. units per g. for the oil assayed, then the assay oil meets the minimum standard for vitamin D potency.[426]

MAXIMUM STANDARD. If the product of $\left(\frac{R}{A}\right)$ × (units per g. of vitamin D contained in the U.S.P. Vitamin D Reference Standard) is equal to or less than the maximum standard in U.S.P. units per g. for the oil assayed, then the assay oil meets the maximum standard for vitamin D potency.[426]

VITAMIN E (TOCOPHEROLS)

The essentiality of vitamin E for optimum nutrition has been demonstrated in more than fifteen vertebrate species by restricting the dietary intake of this vitamin and noting deficiency symptoms. The discovery of vitamin E in 1922 was based upon experiments of H. M. Evans, of the University of California, showing that for normal reproductive function rats required a new fat-soluble factor present in crude vegetable oils and subsequently identified as isomers of tocopherol. Later studies showed that this new vitamin was also essential for normal muscle metabolism, to maintain the integrity of the central nervous system and the vascular system, and for a variety of other physiological functions. The pathology of the symptoms of vitamin E deficiency has been described in great detail, particularly by Pappenheimer and Goettsch and by Mason. However, the exact biochemical mechanism whereby vitamin E functions in diverse metabolic processes in the body is still unknown. This and the direct experimental production and recognition of vitamin E deficiency in human subjects constitute two of the most important problems in the field of vitamin E research. Estimates of the human requirements for this vitamin are based upon indirect evidence, such as the mean daily per capita consumption of vitamin E and the similarity of tissue pathology seen in premature infants and adults suffering from faulty fat absorption to that seen in vitamin E deficient animals.

Physiological and Clinical Aspects of Vitamin E. Not all of the tocopherol in foods is completely available when ingested. The α form is more efficiently absorbed than the non-α-tocopherols. Tocopherols in cereal grains, leafy vegetables, and practically any natural foodstuff are bound in such a way that during passage through the digestive tract some of the tocopherol is excreted in the feces. Balance studies in normal human beings show as much as 60 per cent of ingested α-tocopherol appearing in the feces. Conditions interfering with fat absorption (biliary diseases, pancreatic insufficiency, mineral-oil ingestion, etc.) further reduce the amount of this vitamin absorbed. No free or conjugated tocopherol is excreted in the urine. Both tocopherol and tocopherol esters, when ingested, appear in the blood and are transported as the free form.

α-Tocopherol is deposited in all tissues. The pituitary and adrenal glands have especially high concentrations of tocopherol, over 30 mg. per cent, but the bulk of the body stores of tocopherol is in the adipose tissues and muscles.

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[426] The U.S.P. Unit of vitamin D is defined as the activity of 0.025 μg. of vitamin D_3, contained in the U.S P. Vitamin D Reference Standard.

Vitamin E deficiency symptoms develop in different tissues and at different rates depending upon species. The rabbit and lamb show muscular lesions and paralysis within a few weeks on a tocopherol-free diet. The rat develops testicular degeneration and the chick shows softening of the brain (encephalomalacia) within six weeks. However, the lamb, rabbit, and herbivores in general eventually develop reproductive dysfunction; the rat shows muscle dystrophy and paralysis in about 12 to 18 months; and the chicken eventually develops both muscular and reproductive abnormalities. No common denominator of physiological function of α-tocopherol or of reasons for species specificity and tissue susceptibility has been established; this makes it necessary to consider separately the physiology of individual tissues and organs.

Complex biochemical changes in the muscle tissue in chronic vitamin E deficiency are followed by histological lesions characteristic of muscular dystrophy. The muscle shows either a gradual necrosis and replacement of muscle fibers with fat and connective tissue, or an explosive form of dystrophy involving larger portions of the muscle and accompanied by edema, leukocytic infiltration, and fragmentation of muscle fibers. The former is typified by the muscular paralysis which develops in the adult hamster and has its histological counterpart in humans in progressive muscular dystrophy and dermatomyositis. The explosive type of syndrome is exemplified by paralysis in suckling rats late in lactation and by muscle dystrophy in guinea pigs and rabbits. Heart muscle is affected like skeletal muscle by vitamin E deficiency and rats, rabbits, sheep, and cows have all shown typical lesions of degeneration, pigment deposition, electrocardiogram changes, and functional failure. Vascular abnormalities develop during the production of vitamin E deficiency in all species studied. The dog in particular, when subjected to a high-fat diet combined with kidney injury, develops a reversible arteritis resembling that seen in human arteriosclerosis.

Demyelinization, gliosis, and distortion of the axon pattern in the spinal cord are seen in vitamin E deficient rats, giving rise to hypalgesia and progressive paresis.

The vitamin E deficient male animal shows testicular atrophy and sterility due to irreversible degeneration of the seminiferous tubules and to failure of spermatogenesis. In the vitamin E deficient female, a reversible type of reproductive dysfunction occurs. Ovarian function is normal but uterine physiology is disturbed. There is partial failure of implantation, and those fertilized ova successfully implanted grow and develop only to a certain stage, at which time the fetuses show generalized hemorrhage, die, and are aborted or resorbed. Administration of α-tocopherol during the first half of gestation permits normal fetal development and parturition. This reversibility of reproductive function in the female rat serves as the basis for the bioassay method most generally used to evaluate vitamin E potency.

The normal resistance of red blood cells to rupture, both *in vivo* and *in vitro*, is markedly reduced in vitamin E deficiency. Administration of α-tocopherol to the patient or addition of α-tocopherol to the *in vitro* system returns the resistance of the erythrocytes to normal. This improvement in function of red blood cells by α-tocopherol serves as the basis for a bioassay for vitamin E activity.

The finding that vitamin E metabolism plays an essential role in cardiovascular pathology in lower animals has resulted in attempts to employ tocopherols therapeutically. Cases of angina pectoris, coronary insufficiency, peripheral vascular diseases, etc. have been reported by some investigators to improve under α-tocopherol therapy, but others have failed to observe any benefit in similar clinical trials.

Fatal massive liver necrosis occurs in animals maintained on diets low in vitamin E and sulfur-containing amino acids. Under conditions in which the intestinal flora of the animal are eliminated (germ-free environment, antibiotic treatment), liver necrosis does not develop but the animal dies from lung hemorrhage. α-Tocopherol administration prevents both the liver necrosis and the lung hemorrhage. Possibly this prevention of liver injury by α-tocopherol is merely an example of a physiologic role of α-tocopherol whereby environmental, dietary, and drug stresses are combatted.

Some evidence, both experimental and clinical, points to a sparing effect of α-tocopherol upon hormone action, particularly insulin and the sex hormones. This is considered a result of α-tocopherol exerting an antioxidant or sparing action upon the hormone metabolism. Various pathological states in poultry and livestock have been shown to be aspects of vitamin E deficiency and to respond to administration of tocopherols. Among them are "crazy-chick disease," "enlarged hock disease" of turkey poults, "white muscle disease" of calves and colts, "stiff lamb disease," and steatitis or "yellow fat disease" of mink.

REQUIREMENT. The need for vitamin E has been established qualitatively for a variety of animal species. However, quantitative requirements, either minimal or optimal, for various animals including man, have not been determined.

Diets of good quality consumed by healthy persons in good economic circumstances average between 10 and 25 mg. α-tocopherol daily. Reducing diets and some therapeutic-type diets furnish as little as 4 mg. per day. From a curve relating vitamin E requirements for laboratory animals to (body weight)$^{0.7}$, the value for a 70-kg. animal may be extrapolated. An estimated requirement for humans of 30 mg. D-α-tocopherol is thus derived. From a consideration of the results of these two approaches, the human requirement may be estimated as 15–25 mg. α-tocopherol daily.

BIOPOTENCY. α-Tocopherol and various of its derivatives have different relative potencies depending upon the procedure used for bioassay. Esterified α-tocopherol is more potent by the rat bioassay (in which suboptimal amounts are fed) than an equivalent quantity of free α-tocopherol, probably because of the relative instability of the latter in the intestinal tract (see Table, p. 1270). Tests on human infants and adults with therapeutic doses fail to show this superiority of the ester form. Hence the National Formulary X considers 1 mg. of synthetic α-tocopheryl acetate

RELATIVE BIOPOTENCY OF VARIOUS FORMS OF VITAMIN E

Compound	*Molecular Weight*	*Relative Biopotency**	*International Units per Mg.*
Natural			
D-α-Tocopherol	430.7	101	0.92
D-α-Tocopheryl acetate	472.8	136	1.36
D-α-Tocopheryl acid succinate	530.8	136	1.21
D-α-Tocopheryl disodium phosphate	554.7	13	0.10
Synthetic			
racemic-α-Tocopherol	430.7	75	0.68
" -α-Tocopheryl acetate (International Standard)	472.8	100	1.00 (by definition)

* Based on feeding equivalent amounts of α-tocopherol.

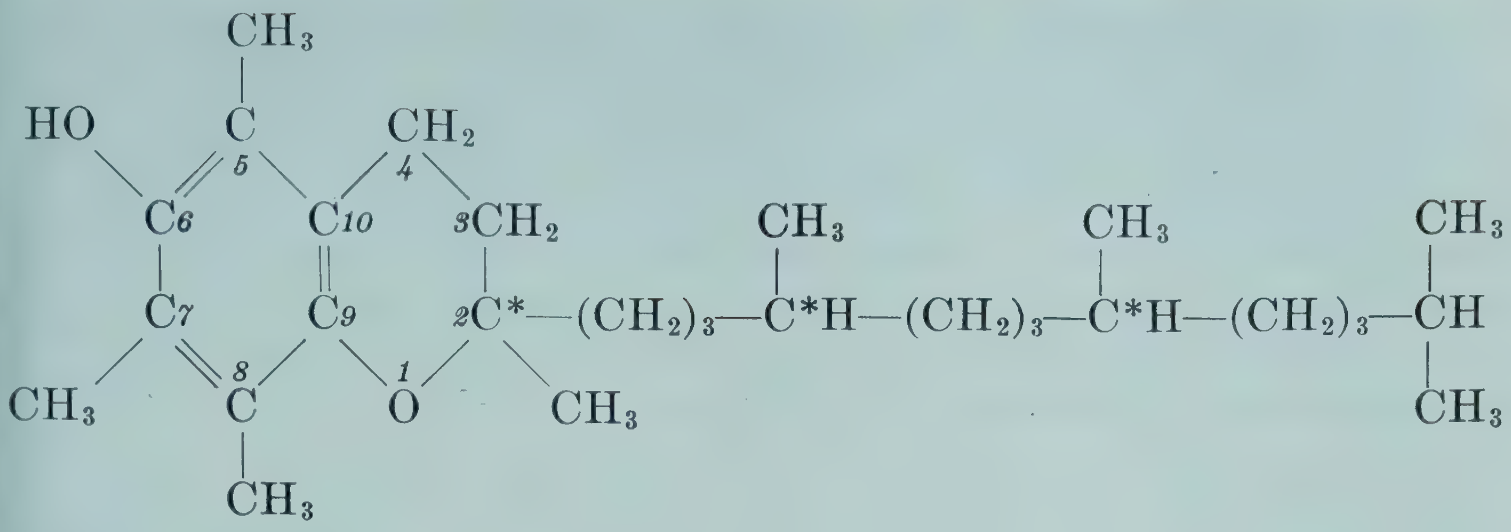
equal to 1.0 I.U. and 1 mg. of natural α-tocopheryl acetate equal to 1.36 I.U. The free α-tocopherols, on the basis of their molecular weight, have a biopotency of 1.1 I.U. per mg. for the synthetic and of 1.49 I.U. per mg. for the natural form.

PHYSIOLOGICAL ANTIOXIDANT ACTIVITY. The fact that protoplasm is an aqueous-lipide emulsion in which metabolic reactions occur at interfaces immediately poses the problem of properly stabilizing the lipide phase. α-Tocopherol is the only naturally occurring, fat-soluble food antioxidant which accompanies lipide through the intestinal tract and during its transport via blood or lymph to storage depots or metabolic sites. Other antioxidants are eliminated during absorption or in transit, and α-tocopherol emerges as the only physiological fat-soluble antioxidant.

The amount of α-tocopherol deposited in the tissues is proportional to the quantity ingested. In certain species—for example, turkeys and pigs—the concentration of α-tocopherol in tissue lipides is so low that turkey carcass fat and lard oxidize and become rancid even under conditions of low-temperature storage. The cow excretes a proportional amount of ingested or stored α-tocopherol into her milk, and whenever the level falls below 20–30 μg. per g. milk fat, the milk has poor stability and off-flavor develops.

Chemistry of the Tocopherols. The four compounds in vegetable oils possessing vitamin E activity have been isolated and their structures proved. They were named *tocopherols* (Greek, "young-bearing"), and found to differ only in the extent of methylation of the chroman nucleus. α-Tocopherol was synthesized by L. I. Smith and by Karrer, independently, in 1938. The structure of α-tocopherol is shown on p. 1271 with those carbon atoms marked where asymmetry may occur, thus giving rise to isomers. Natural α-tocopherol is the D form whereas synthetic vitamin E consists of a mixture of isomers usually designated as racemic DL-α-tocopherol.

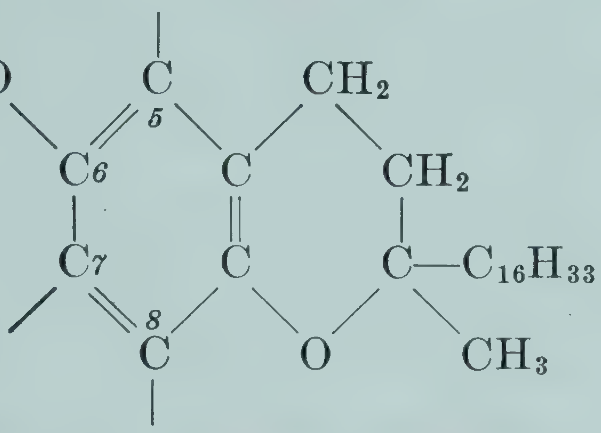
The other tocopherols, β-, γ-, and δ-tocopherols, have been synthesized and characterized. However, they are considered to be less important, since they are less effective physiologically than α-tocopherol. The structural differences of the various tocopherols are shown in the follow-



α-Tocopherol

* Points of asymmetry. Natural-occurring α-tocopherol is the D form, whereas synthetic α-tocopherol is a mixture of isomers.

ing scheme. The β form has about one-third the biopotency of α-tocopherol, the γ form about one-twelfth, and the δ form about one-hundredth.



The Tocopherol Nucleus

Name	*Substituents at Positions* 5	7	8
α-Tocopherol = Trimethyl derivative	CH_3—	CH_3—	CH_3—
β- " = Dimethyl "	CH_3—	H—	CH_3—
γ- " = Dimethyl "	H—	CH_3—	CH_3—
δ- " = Methyl "	H—	H—	CH_3—

α-Tocopherol is easily destroyed by oxidation. Esterification with acetic, succinic, phosphoric, and other acids is commonly practiced to stabilize the vitamin. These esters have essentially the same potency as α-tocopherol except that under certain test conditions they bioassay higher than the free tocopherol because of their enhanced stability.

Other changes in the molecule, such as alteration of the side chain, etherification at position 6, or substitution of the ring methyl groups, markedly reduce or completely eliminate vitamin potency. Oxidation of α-tocopherol, which progresses through an epoxide intermediate, a hydroquinone, and a free radical semiquinone to tocopherol quinone, destroys the vitamin E potency for most animal species. However, the epoxide intermediate is physiologically active for the rat, the tocopherol hydroquinone will prevent or cure muscle degeneration in the hamster and the final oxidation product, tocopherol quinone, has vitamin E activity for the rabbit.

Occurrence and Distribution of Vitamin E. Vegetable-seed oils, particularly those of wheat, soybean, cotton, and corn, are the richest natural sources of tocopherols (approximately 0.5 to 3.0 mg. per g.). These are used as source materials for the manufacture of vitamin E concentrates and pure natural α-tocopherol preparations. Margarine made from hydrogenated vegetable oils is one of the best food sources of α-tocopherol. Dairy products supply relatively low and variable amounts of α-tocopherol. Colostrum, both human and bovine, contains levels of α-tocopherol ten times higher than later milk. This is important because the newborn infant or calf has a poor endowment of tocopherol in its tissues. The total amount of tocopherol in the newborn infant is about 20 mg., less than the amount eaten daily by a well-fed adult. Cow's milk is only from one-fourth to one-half as potent as human milk in α-tocopherol content. This fact should be considered when cow's milk is substituted for human milk in infant feeding. Hydrogenation and refining processes do not completely destroy this vitamin. Other foods of vegetable origin, such as lettuce, celery, and sweet potato, while rich in tocopherol on the basis of their fat content, make relatively minor contributions of vitamin E to the diet.

Foods of animal origin are generally poor sources of α-tocopherol unless one excepts meat cuts from animals fed rations high in tocopherol just prior to slaughter. Milk, butter, cheese, other dairy products, and eggs are similarly quite low in α-tocopherol content.

DETERMINATION OF VITAMIN E

CHEMICAL METHODS

Analysis of biological materials for α-tocopherol content is complicated because of the presence of non-α-tocopherols, carotenoids, and many other extraneous materials which possess reducing power and which must be corrected for, or eliminated, prior to colorimetric measurement. Another difficulty is the problem of extracting, saponifying, and concentrating the small quantities of this vitamin present without oxidative destruction and loss.

Analyses of foodstuffs, blood, and other tissues for α-tocopherol require different steps of extraction, concentration and purification.

The colorimetric measurement of pure tocopherols with ferric chloride-dipyridyl reagent is precise but nonspecific. Any compound capable of reducing ferric to ferrous ions, which then react with dipyridyl to form a red color, would be measured as tocopherol. However, for the determination of tocopherol in purified extracts, or in pure standard solutions of α-tocopherol, the following procedure may be used:

Ferric Chloride-Dipyridyl Method (Emmerie-Engel Reaction).

Procedure. **Prepare a solution in absolute ethanol containing from 10 to 25 mg. tocopherol per 100 ml. Transfer 2 ml. of this solution to an opaque, glass-stoppered flask. Add 1.0 ml. ferric chloride reagent and 1.0 ml. 0.5 per cent dipyridyl in absolute ethanol (redistilled from 0.02 per cent alkaline potassium permanganate). Swirl to mix. Add 21 ml. of absolute ethanol and shake vigorously. After $9\frac{1}{2}$ minutes from the addition of ferric chloride**

reagent (0.1 g. ferric chloride dissolved in 50 ml. absolute ethanol), transfer an aliquot to a spectrophotometer or colorimeter cell. Determine the absorbancy at 520 mμ against absolute ethanol as a blank, at exactly 10 minutes after addition of the ferric chloride. Prepare a control sample exactly as described except for the use of 2 ml. absolute ethanol for the 2 ml. tocopherol solution. Again, after 10 minutes determine the absorbancy at 520 mμ against absolute ethanol as a blank.

The difference between the absorbancies of the control sample and the test sample is converted to "mg. tocopherol" by reference to a standard curve.

A good alternative procedure consists in using ceric sulfate as the oxidizing reagent and titrating the tocopherol-containing extract using diphenylamine or ferroin as the indicator.

Saponification (*For Extracts Containing Tocopherol Esters or α-Tocopheryl Acetate Standards*). Weigh a sample containing 150–300 mg. tocopherol ester into a 250-ml. glass-stoppered actinic glass flask. Use of rubber stoppers may introduce interfering reducing substances, and exposure to light causes destruction of tocopherol. Add 50 ml. absolute ethanol, fit with a reflux condenser, and reflux for at least 1 minute to remove all traces of air from the system. Add carefully and slowly through the condenser 1 g. KOH to the refluxing solution. Reflux for 20 minutes. Without cooling, add 2 ml. HCl dropwise. Cool to room temperature and transfer to a 500-ml. separatory funnel, rinsing the flask with 100 ml. water and 100 ml. freshly distilled, peroxide-free ethyl ether. Shake vigorously. Allow the layers to separate. Extract the aqueous layer twice more with 50-ml. portions of ethyl ether. Wash the combined ether extracts with four 100 ml. portions of water. Evaporate the ether solution on a water bath, in vacuum or under streams of nitrogen, to less than 10 ml.; then to dryness without heat. Immediately dissolve the residue in absolute ethanol, transfer to a 250-ml. volumetric flask and dilute to volume.

Alternatively, the labile tocopherol may be protected during the saponification step by using antioxidants, such as pyrogallol or acetyl *p*-aminophenol.

Analysis of Biological Tissues (*Method of Quaife and Dju*[427]). Practically all of the tocopherol in human and animal tissues is α-tocopherol. A specimen is taken and frozen solid in dry ice. It is pulverized, sieved, sampled, and weighed in the frozen state. The sample is then extracted with hot ethanol for 24 hours, transferred to purified petroleum ether, molecularly distilled, and hydrogenated. The extract is then assayed by the ferric chloride–dipyridyl method.

Blood serum or plasma is easier to analyze for tocopherol content since the molecular distillation step may be omitted.[428]

Analysis of Foods (*Method of Quaife and Harris*[429]). The procedure for foods, particularly vegetable foods, is similar to that used for animal tissues except that a dehydration step is used to effect some concentration of tocopherol, and the ethanol extraction time is only two hours. Final colorimetric estimation of tocopherol with ferric chloride dipyridyl reagent measures total tocopherols, and some procedure for obtaining only the α-tocopherol content is required. In foods where the tocopherol concentration is low, γ- + δ-tocopherol concentration is measured by diazotization with dianisidine.[430] (β-Tocopherol is not measured, but this form occurs only in wheat products.) The α-tocopherol content is obtained by subtracting the combined value for γ- + δ-tocopherols from the total tocopherol value.

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[427] Quaife and Dju: *J. Biol. Chem.*, **180**, 263 (1948).

[428] Quaife and Harris: *J. Biol. Chem.*, **156**, 499 (1944); Quaife and Biehler: *J. Biol. Chem.*, **159**, 663 (1945).

[429] Quaife and Harris: *Anal. Chem.*, **20**, 1221 (1948); Harris, Quaife and Swanson: *J. Nutrition*, **40**, 367 (1950).

In preparations where at least 0.5 mg. of β- + γ- + δ-tocopherols are available, analysis of their total content is preferably done by the nitroso method.[431] This value for the combined amount of all non-α-tocopherols, subtracted from the total tocopherol value determined by the ferric chloride-dipyridyl method, has been used to give a measure of the α-tocopherol content.

Separation, isolation, and direct measurement of α-tocopherol in extracts of biological materials has been done semiquantitatively by means of paper chromatography. Brown[432] has reported separation of the tocopherols, using petrolatum-coated paper strips and 75 per cent ethanol as solvent, and has estimated the individual tocopherol content of various animal and vegetable tissues.

BIOLOGICAL METHODS

Evans Resorption-Gestation Method, Modified by Mason and Harris.[433] This bioassay is based upon the ability of α-tocopherol, administered to vitamin E deficient female rats during the first half of pregnancy, to prevent fetal death and resorption. Female rats with low vitamin E reserves are raised on the following vitamin E low diet:

Casein, commercial unextracted	20	per cent
Carbohydrate, starch, or sucrose	56	"
Fat, lard, or olive oil	10	"
Salt mixture	4	"
Brewer's yeast, dried	10	"
Vitamins A and D, added to fat component to supply 10 units vitamin A and 1 unit vitamin D per gram of diet		

When the females are 150 g. in weight, they are mated with normal males. Vaginal smears are made daily until sperm (sign of positive mating) are observed. The pregnant females are placed in individual cages and assigned at random to control or test groups.

Groups of six to ten rats are set up on four or five levels of both the unknown material being assayed and the standard. (The International Vitamin E Standard is a preparation of DL-α-tocopheryl acetate in olive oil. The biological activity of 1 mg. of this DL-α-tocopheryl acetate = 1 International Unit of vitamin E.)

The vitamin E supplement is fed orally in five daily doses prior to the tenth day of pregnancy. On the twentieth day of pregnancy the rats are killed and autopsied. The uterus is inspected for live, dead, and resorbed fetuses. Only those animals with four or more implantation sites are considered. A "positive" response is recorded for animals with one or more viable fetuses, and a

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[430] Quaife: *J. Am. Chem. Soc.*, 66, 308 (1944).

[431] Quaife: *J. Biol. Chem.*, 175, 605 (1948).

[432] Brown: *Biochem. J.*, 52, 523 (1952).

[433] Mason and Harris: *Biol. Symposia*, 12, 459 (1947).

"negative" for those with no living fetus. A dose:response curve is constructed relating dose (logarithm) to response (litter efficiency, i.e., percentage of positive responses expressed as probits[434]) for both the standard and the unknown. These are compared at the 50 per cent end point (the Median Fertility Doses) and the potency of the unknown is expressed in terms of International Units of vitamin E.

***Rose-György Erythrocyte Hemolysis Test.*[435] Adult rats, two to three months old, have red blood cells which are normally resistant to the hemolytic action of dialuric acid, hydrogen peroxide, and similar reagents. Changing the animals to a vitamin E low diet for even a few days causes the red cells to become susceptible to hemolysis. Administering various levels of α-tocopherol, either prophylactically or curatively, to the rats during the depleting period and measuring the degree of red cell hemolysis under arbitrary standard conditions gives a satisfactory bioassay procedure.**

***Procedure.* Rats receiving various levels of pure α-tocopherol as standard and other groups receiving similar levels of tocopherol in the unknown material, both within the range to furnish 0.1 to 0.4 mg. α-tocopherol per day, are used. The supplements are fed daily for two weeks and on the fifteenth day blood samples are taken and tested. Obtain blood from tail of rats, 10 to 12 drops in a graduated 15-ml. centrifuge tube containing 2 ml. anticoagulant (equal parts of 0.9 per cent NaCl and 1 per cent sodium citrate solutions). Centrifuge and discard supernatant fluid. Dilute the red cells to a 5 per cent suspension with 0.9 per cent NaCl solution.**

Pipet 0.25 ml. erythrocyte suspension into each of three test tubes or colorimeter tubes. To one (control) add 4.75 ml. of saline-buffer solution;[436] to tube 2 add 4.75 ml. H_2O, which hemolyzes red cells completely; to tube 3 add 4.45 ml. saline-buffer solution[436] and 0.30 ml. dialuric acid solution.[437] Mix and incubate for 30 minutes at 37° C. Allow to stand at room temperature for at least two hours, invert tube, centrifuge, and measure in the colorimeter the red color from the hemolyzed cells.

CALCULATION. The reading of the control sample is subtracted from readings of both the test sample and the completely hemolyzed sample. The percentage of hemolysis is calculated as the ratio of the corrected test-sample reading $\times 100$, to the corrected hemolyzed-sample reading.

Human infants, especially premature, have red blood cells which are hemolyzed by dilute hydrogen peroxide. Administration of α-tocopherol, either *in vivo* or *in vitro*, prevents this hemolysis.[438]

Other bioassays have been developed and used to a limited extent. For example, young male rats raised for three months or more on vitamin E deficient diets supplemented with various levels of α-tocopherol, 0.0 to 1.0 mg. daily, show testis weight proportional to the dose of α-tocopherol. The severity of testicular degeneration as seen histologically is inversely proportional to the dose of tocopherol.

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[434] Converting percentage responses to probits (a mathematical convention), by reference to tables, results in a straight-line dose: response curve instead of a sigmoid-shaped curve. This makes it easier to derive a median value from the data (Bliss: *Ann. Applied Biol.*, **22**, 134, 1935).

[435] Rose and György: *Proc. Soc. Exptl. Biol. Med.*, **74**, 411 (1950).

[436] *Saline Phosphate Buffer*, pH 7.4: 0.2 M KH_2PO_4, 50 ml.; 0.2 M NaOH, 39.34 ml.; and water up to 200 ml. Mix with equal volume of 0.9 per cent NaCl.

[437] *Dialuric Acid Solution.* 1 mg./ml. saline phosphate buffer solution (equal parts of phosphate buffer and 0 9 per cent NaCl solution). Since dialuric acid is easily oxidized, the solution should be prepared immediately before use.

[438] György, Cogan, and Rose: *Proc. Soc. Exptl. Biol. Med.*, **81**, 536 (1952).

Rabbits fed a vitamin E deficient diet develop muscle dystrophy in about three weeks which is characterized by creatinuria. In the early and reversible stages of the dystrophy the creatine excretion can be reduced temporarily to normal by single doses of vitamin E. The time elapsed from dosing to the return of the creatinuria to its initial high level is proportional to the dose of tocopherol.

VITAMIN K

In the late 1920's, Dam[439] observed a hemorrhagic syndrome in chicks raised on a diet low in lipides which failed to respond to rich sources of the known vitamins. The disease is associated with a reduction in clotting power of the blood, hemorrhages occurring especially in regions exposed to trauma. In 1935, Dam advanced the claim that this syndrome was due to deficiency of a new fat-soluble vitamin in green leaves, which he called vitamin K (for Koagulations). Almquist and Stokstad[440] independently announced a similar claim for a factor present in putrefied fish meal. In 1939, the pure vitamin was isolated from alfalfa by Dam and others[441] and from both alfalfa and putrefied fish by MacCorquodale, Binkley, McKee, Thayer, and Doisy.[442] Physical and chemical differences in the vitamins thus isolated led to the designation K_1 for the vitamin from green leaves and K_2 for that formed by bacterial putrefaction. Investigation of the vitamin K molecule has revealed the presence of a quinoid ring structure, 2-methyl-1,4-naphthoquinone, which alone or substituted in various ways possesses all or part of the physiological activity of the vitamin; thus vitamin K has various natural and synthetic vitamers.

Physiological and Clinical Aspects of Vitamin K. The specific type of hemorrhagic diathesis observed in vitamin K deficiency is due to a lowering of the prothrombin level of the plasma. (For the role of prothrombin in the coagulation of blood, see p. 477.) Hypoprothrombinemia may, however, result from other causes, such as cirrhosis, chloroform poisoning, or Banti's disease, where liver tissue is damaged. Vitamin K is without value in hemophilia, a condition due to a deficiency of thromboplastin and hence a failure of conversion of prothrombin to thrombin. Vitamin K does not alter the clotting time of hypoprothrombinemic blood when added *in vitro;* its function in the body is to facilitate the synthesis of prothrombin in the liver, but it has not been definitely established whether the role of vitamin K is that of a precursor or a catalyst. Prothrombin synthesis occurs in the liver, and a vitamin K "tolerance test" for normal liver function has been proposed. In cases of prothrombin deficiency administration of vitamin K causes the prolonged prothrombin time to return to normal, provided the impairment of liver function has not reached an advanced state.[443] The prolonged clotting time associated with hypervitaminosis A responds to vitamin K administration.[444] Chicks, ducklings, and other birds fed a vitamin K-deficient diet develop severe hemorrhagic lesions both internally and subcutaneously, especially under the wings, on the legs, breast, abdomen, and neck, and in the intestinal tract. The concomitant anemia terminates in death. In the rat, bacterial synthesis of vitamin K_2 in the intestine, especially when coprophagy is possible, usually obscures the effect of a vitamin-K-free diet.

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[439] Dam: *Biochem. Z.*, **215**, 474 (1929); **220**, 158 (1930); *Biochem. J.*, **29**, 1273 (1935).

[440] Almquist and Stokstad: *J. Biol. Chem.*, **111**, 105 (1935); *J. Nutrition*, **12**, 329 (1936).

[441] Dam, Geiger, Glavind, Karrer, Karrer, Rothschild, and Salomon: *Helv. Chim. Acta* **22**, 310 (1939).

[442] MacCorquodale, Binkley, McKee, Thayer, and Doisy: *Proc. Soc. Exptl. Biol. Med.* **40**, 482 (1939).

Simple dietary deficiency of vitamin K is rare, not merely because of the presence of the vitamin in foods, but because intestinal microorganisms, especially of the *coli* group, synthesize the vitamin, which is released upon putrefactive disintegration of the bacterial cells. K avitaminosis in humans is more often associated with impaired absorption of fat (and concomitantly of vitamin K) such as is seen in obstructive jaundice and biliary fistulas, as well as in intestinal disorders like celiac disease and ulcerative colitis. In these conditions, or after removal of biliary obstructions, vitamin K is administered parenterally or orally to prevent or relieve the bleeding tendency. When the flow of bile is obstructed, oral dosage of vitamin K must be accompanied by bile salts to insure its absorption; this may be avoided by the use of water-soluble derivatives of the vitamin. Vitamin K preparations are also used to treat hemorrhagic emergencies (hypoprothrombinemia) resulting from the use of antiprothrombinemic drugs in thromboembolic conditions (e.g., coronary artery thrombosis).

The most common form of vitamin K deficiency is seen in newborn infants, so-called *hemorrhagica neonatorum.* The blood prothrombin level is low at birth; it decreases further during the first few days, rises sharply and then gradually until a normal level is reached between one and two months of age. Whether the initial hypoprothrombinemia is due to poor placental transfer has not been established. It is significant that milk, both human and cow, is a very poor source of vitamin K. The rise in prothrombin after birth is attributed to bacterial synthesis in the intestine. Administration of vitamin K to the mother before parturition results in higher blood prothrombin levels at birth. Direct dosage of the infant with as little as one microgram of the vitamin is also effective.

The minimum daily requirement of vitamin K has not been established. The extremely low prophylactic dose for the infant, and subsequent synthesis in the intestine, indicate that the dietary requirement, if any, is small. One to 2 mg. of the vitamin is capable of correcting most deficiencies, although the therapeutic dose may vary with the severity of the hepatic or intestinal condition. For further discussion of clinical aspects, see p. 1293.

Storage and Distribution of Vitamin K. With the possible exception of the liver, the organs and tissues of the body do not store vitamin K to any significant degree. In the hen, even when the ration is rich in this vitamin, only small concentrations are found in the various organs. Vitamin K is not found in the urine; the high concentration in the feces is due largely to intestinal synthesis.

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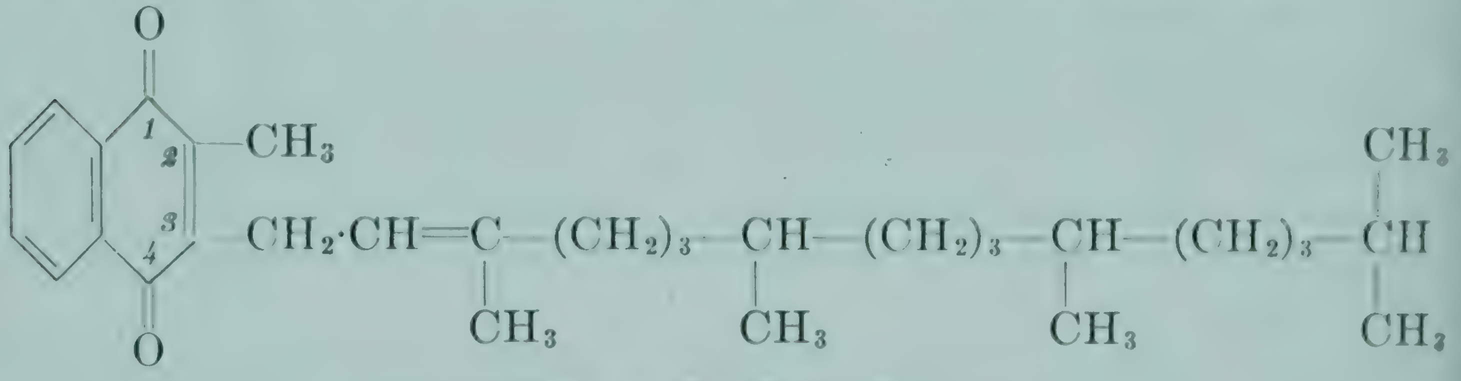
[443] Unger, Weiner, and Shapiro: *Am. J. Clin. Pathol.*, **18**, 835 (1948).

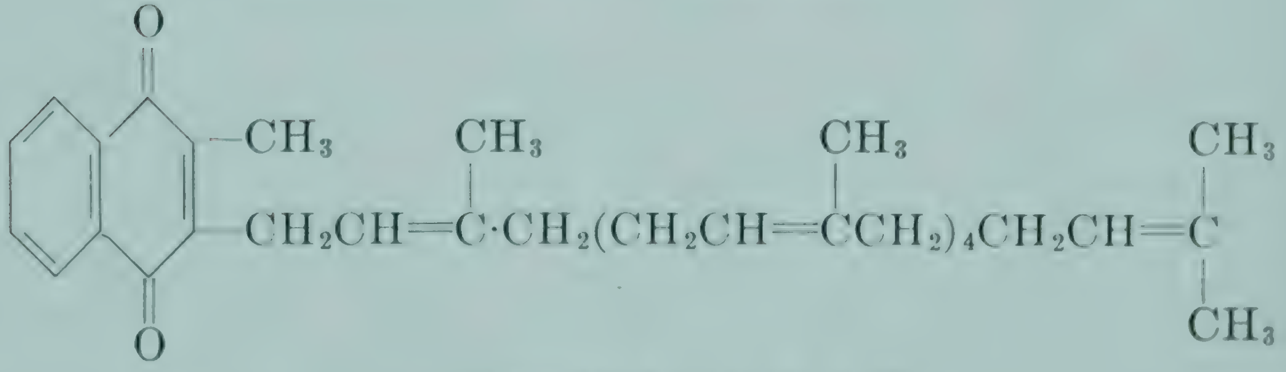
[444] Walker, Eylenburg, and Moore: *Biochem. J.*, **41**, 575 (1947); Quick and Stefanini: *J. Biol. Chem.*, **175**, 945 (1948).

Occurrence of Vitamin K. The principal natural source of vitamin K is the green leaf or other chlorophyll-containing portions of plants; this form of the vitamin (K$_1$) has therefore been named *phylloquinone*. The vitamin is present in high concentration in spinach and alfalfa, while cabbage, cauliflower, seaweed, and carrot tops are good sources. Most seeds, fruits, and roots (including cereals, beans, potatoes, peas) contain little if any vitamin K, although soybean oil, tomatoes, orange peel, and hemp seed are good sources. Milk and eggs are poor sources of vitamin K even when the ration of the cow or hen contains a high level of the vitamin.

Chemistry of Vitamin K. Recognition of the quinoid structure of vitamins K$_1$ and K$_2$ was due to the work of McKee, *et al.*[445] at Washington University, St. Louis, and of Karrer and Geiger.[446] The former group established that these vitamins were derivatives of 2-methyl-1,4-naphthoquinone with substituent groups in the 3 position, K$_1$ containing the phytyl group and K$_2$ a similar but longer (difarnesyl) side chain.



Vitamin K$_1$ (C$_{31}$H$_{46}$O$_2$)
2-methyl-3-phytyl-1,4-naphthoquinone



Vitamin K$_2$ (C$_{41}$H$_{56}$O$_2$)
2-methyl-3-difarnesyl-1,4-naphthoquinone

Vitamin K$_1$ is a light yellow, viscid oil, whereas K$_2$ is a yellow crystalline solid (melting point 54°). Both are soluble in oil and various fat solvents. The vitamins are sensitive to light and, as would be expected from their quinoid structure, are destroyed by saponification. Vitamins K$_1$ and K$_2$ are characterized by ultraviolet absorption maxima at wavelengths of 243, 248, 261, 270, and 328 mμ. The quinoid ring common to these compounds is responsible for their physiological activity. This is shown by the fact that the synthetic compound 2-methyl-1,4-naphthoquinone, to which the name *menadione* has been given, is equally as active on a molar basis as the natural vitamers. Menadione has a molecular weight of 172,

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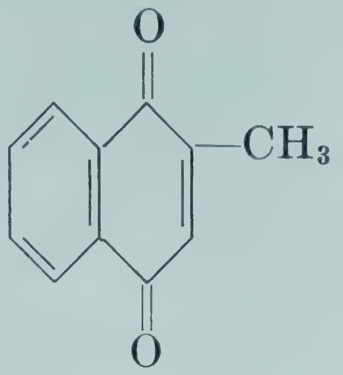
[445] McKee, Binkley, MacCorquodale, Thayer, and Doisy: *J. Am. Chem. Soc.*, **61**, 1295 (1939).

[446] Karrer and Geiger: *Helv. Chim. Acta*, **22**, 945 (1939).



Menadione ($C_{11}H_8O_2$)
2-methyl-1,4-naphthoquinone

whereas the molecular weights of K_1 and K_2 are 450 and 580 respectively. Menadione is a yellow, crystalline compound (melting point 106°), slightly soluble in water and soluble in alcohol, ether, acetone, and glacial acetic acid and in vegetable oils. It is light-sensitive and, in common with other quinones, has a burning taste and is irritating to mucous membranes. The long substituent side chains of the natural vitamins diminish their solubility in water and, at the same time, their taste.

Many active derivatives and homologs of menadione or the natural vitamins have been prepared but always with a diminution or loss of biological potency. Phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone), a constituent of the tubercle bacillus, was early recognized to have slight vitamin K activity. Substitution of the 2-methyl group by hydrogen or various alkyl radicals results in marked loss of activity. Substitution in the 3 position does not cause a serious drop in potency, although a double bond in the position shown for the phytyl group in vitamin K_1 and a long branched side chain are essential in such substituted compounds.

The hydroquinones of vitamins K_1 and K_2 or of menadione may be converted into diacetates, diphosphates, disulfates, etc., which possess lower activity than the corresponding quinones but are less irritating to the mucosa of the mouth and gastrointestinal tract. Nevertheless, certain of these derivatives which, unlike natural vitamin K, are water-soluble and more stable to light and air have been employed clinically. Important among these is menadione sodium bisulfite, a white crystalline powder containing 49 per cent menadione.

DETERMINATION OF VITAMIN K

Physical or chemical procedures for the estimation of vitamin K activity are complicated by the presence of interfering substances (including vitamins A and E), the different forms in which vitamin K occurs in nature, and their susceptibility to destruction by light, heat, and air, especially during the saponification and extraction steps. The method of Scudi and Buhs[447] involving reduction of the quinones by catalytic hydrogenation, and subsequent reaction of the hydroquinones with 2,6-dichlorophenol indophenol, appears to offer the best approach to the determination of vitamin K in extracts of natural materials including blood and plasma.

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[447] Scudi and Buhs: *J. Biol. Chem.*, **141**, 451 (1941); **143**, 665 (1942); **144**, 599 (1942).

The extent of the reaction is measured in a photoelectric colorimeter. The use of hydrosulfite has been recommended to avoid destruction of the vitamin during saponification.

ASSAY FOR VITAMIN K

Biological Method of Almquist:[448] ***Principle.*** The assay is based on the relative doses of assay material and of standard menadione required to restore the prothrombin clotting time of the blood of vitamin K depleted chicks.

Procedure. **One- or two-day-old chicks are placed in heated (90° to 95° F.) battery brooders with wire-mesh floors. To prevent consumption of vitamin K from bacterial synthesis, food and water should be provided through apertures outside the cage, and moist or soiled food should be discarded. The following ration is fed *ad libitum:***

Sardine meal (ether extracted)	17.5
Dried brewers' yeast (ether extracted)	7.5
Ground polished rice	72.5
Codliver oil	1.0
Calcium carbonate	0.5
Manganous sulfate	0.005

After 10 to 14 days, when the clotting time of the blood of 5 per cent of the chicks is 15 minutes or more, divide them into groups of 12. (Determine clotting time as follows: Withdraw a few drops of blood from clean cut of exposed wing vein. Place in small vials and shake in water bath at 38.5° to 39° C. Time from withdrawal of blood to formation of a firm clot.)

Maintain one group on basal ration as negative controls, at least two groups on different dosages of U.S.P. menadione, and at least one on each product to be assayed. Administer doses orally in 0.1 ml. water or ethyl laurate, depending on solubility. Open mouth of chick by applying pressure at the corners so that the dose may be given with a tuberculin syringe (fitted with a blunt-edged needle) well down in the throat. Do not permit access to food or water for one-half hour after dosage. Repeat dosage for four days at 24-hour intervals.

Determine prothrombin clotting time on all chicks 24 hours after administration of last dose, as follows: Place 0.2 ml. of 0.1 M sodium oxalate in short, narrow tubes graduated at 2 ml. Introduce 2 ml. blood from each chick into a tube. (Blood may be obtained by decapitation with scissors and directing flow into tube with fingers.) Shake thoroughly. Pipet 0.1-ml. portions into small flat-bottomed vials (15 × 50 mm.). Add 0.2 ml. of clotting agent[449] and start timing with stopwatch. Place vials in thermostatically controlled water bath at 38.5° to 39° C. so that they are tilted at an angle of 45° in a device to permit moving them to a vertical position once per second. When gelatinous film (clot) covers bottom of vial, stop watch and record

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[448] Almquist: *J. Assoc. Off. Agr. Chem.*, **24**, 405 (1941); also *Methods of Analysis of the A.O.A.C.*, 6th ed. Washington, D.C., 1945. See also Almquist: *Biological Symposia*, **12**, 508 (1947).

[449] *Preparation of Clotting Agent.* Maintain 4 or 5 chicks on a practical ration containing at least 5 per cent dried alfalfa. (Avoid contamination of vitamin K deficient ration.) Kill one bird by bleeding. Excise 10 g. of breast muscle and grind with sand and 10 ml. of 0.85 per cent NaCl. Centrifuge and filter through coarse paper. Store in refrigerator no more than a few days. Dilute to 200 ml. with 0.85 per cent NaCl and mix with 200 ml. 0.025 M $CaCl_2$. This clotting agent should clot blood of normal chicks in 20 to 30 seconds. If it does not, modify concentration until prothrombin time falls within this range.

"prothrombin time." Duplicate test on each blood sample until results agree within 2 seconds.

CALCULATION. Plot the mean prothrombin time (M.P.T.) against log micrograms of menadione for reference groups and draw best-fitting straight line. Interpolate equivalent dosage of assay material. (1 μg. menadione = 1 A.O.A.C. unit of vitamin K activity.)

THE PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

The vitamin content of a food, or of the diet as a whole, as determined by chemical or microbiological assay, does not always constitute a reliable indication of the actual amount of utilizable vitamin consumed. Failure of foods to be completely digested and absorbed from the intestinal tract, *in vivo* destruction due to oxidation or incompatibilities among the dietary constituents, abnormal pH conditions due to gastric hypoacidity or the use of antacids—these are among the many factors which influence the physiological availability of the vitamins. Vitamins often exist in nature in firm union with protein or other compounds, necessitating hydrolysis prior to chemical or microbiological assay to a degree not duplicated in the gastrointestinal tract. Animal assays are a better measure of physiologically available vitamins, although they do not always simulate conditions in man. For example, assay materials sometimes have to be subdivided or dissolved in order to feed the small doses required by animals; moreover, purified basal rations deviate considerably from the diet of man.

These factors emphasize the desirability of a human assay technique for determining physiologically available vitamins in foods and other sources and for determining the effect of various conditions which influence their availability. A highly reproducible procedure developed by Melnick, Hochberg, and Oser,[450] is based on the fact that normal human subjects subsisting on a complete diet excrete the water-soluble vitamins in the urine in direct proportion to the quantity consumed above the adequate basal level. These vitamins may be excreted either unchanged or as derivatives. The linear relationship of excretion to dosage is established for a group of experimental subjects by feeding the vitamins in pure solution, the form in which they are most completely available. Fig. 292 illustrates a series of such urinary excretion studies for thiamine, riboflavin, niacinamide, and ascorbic acid. To determine the physiologically available vitamin content of an unknown material, the experiment is repeated, feeding an amount of the test food furnishing (according to chemical or microbiological analysis) a critical dose of the vitamin in question. The relation between the extra urinary excretion of the vitamin in the test dose and in the pure solution constitutes the index of physiological availability. For example, if under the test conditions three-quarters as much extra thiamine (i.e., above the basal excretion level) is found in the 24-hour urine following the ingestion of a given food as was excreted following the ingestion of an equivalent amount of thiamine in the form of the pure solution, the physiological availability of the thiamine in the test food is said to be 75 per cent.

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[450] Melnick, Hochberg, and Oser: *J. Nutrition*, **30**, 67 (1945).

The illustrations of the application of this technique which follow are taken from the work of Melnick, Hochberg, and Oser.[450] The method may be applied only to those vitamins normally excreted via the urinary tract, which of course excludes the fat-soluble vitamins.

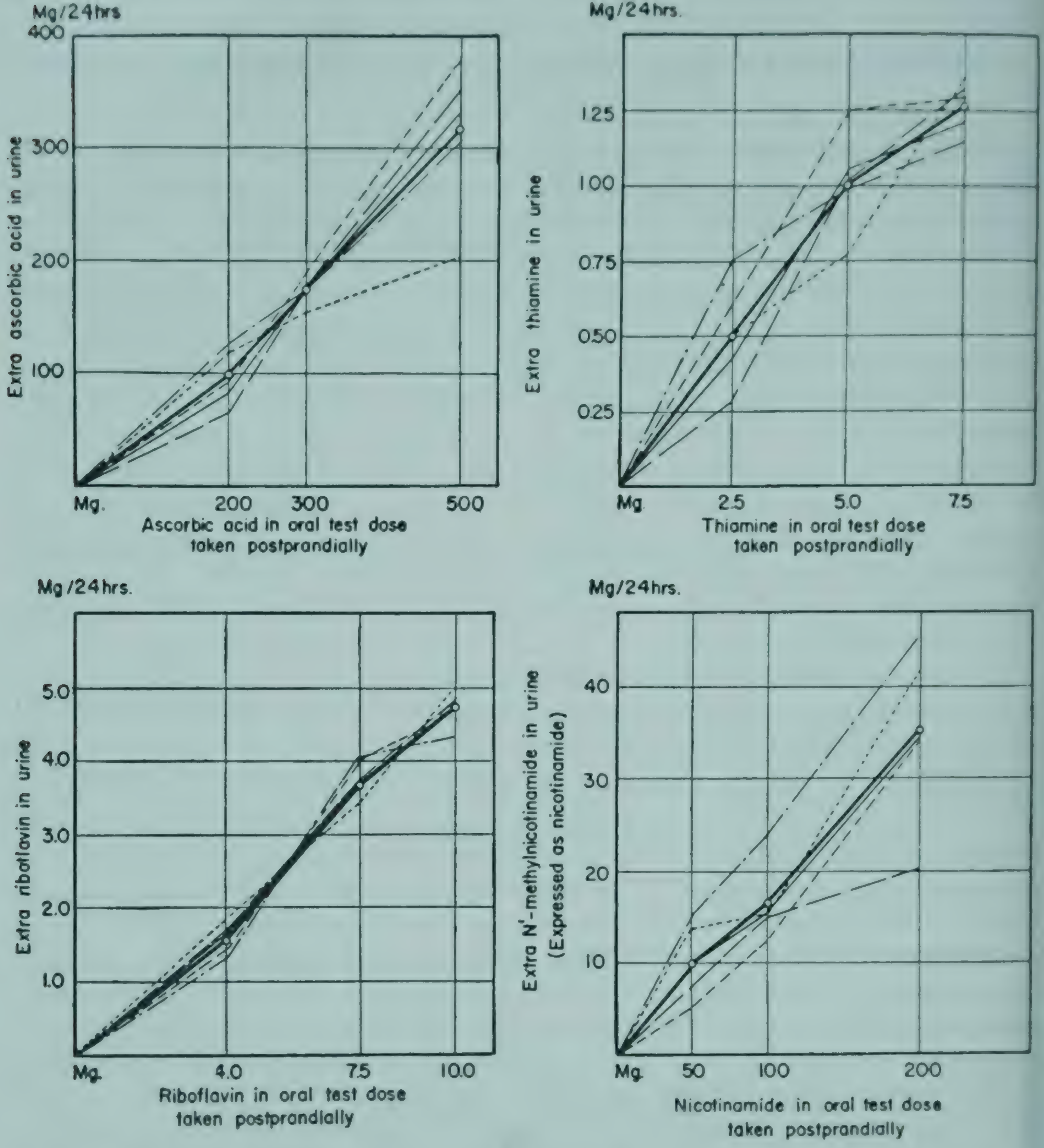

FIG. 292. THE LINEAR RELATIONSHIP BETWEEN DOSAGE WITH THE WATER-SOLUBLE VITAMINS ASCORBIC ACID, THIAMINE, RIBOFLAVIN, AND NICOTINAMIDE, AND THE EXTRA URINARY EXCRETION OF THE VITAMINS (OR DERIVATIVE).

The test doses in aqueous solution (pH 3.0) were taken orally immediately after dinner. The fine lines represent the responses of the individual subjects, whereas the heavy line indicates the average responses.

Any suitable method may be employed for the analysis of the urine for the vitamin and its metabolites. It is worth noting that thiamine is excreted in the urine as the free, unphosphorylated vitamin; ascorbic acid is largely, but not entirely, in the reduced state, so that reduction of the dehydro form is necessary; nicotinic acid is excreted principally as the metabolite N^1-methylnicotinamide, and vitamin B$_6$ chiefly as pyridoxic acid.

Determinations of the physiological availability of the vitamins employing this procedure have been reported for pharmaceutical tablets in which the vitamin ingredients are protectively coated to insure their stability; for the thiamine in live yeast cells; for ascorbic acid as influenced by the presence of copper or ascorbic acid oxidase; for thiamine as influenced by the presence of the antithiamine factor in raw fish such as clams; and for the B vitamins in the presence of adsorbing agents.

Determination of the Physiological Availability of Thiamine in Live Yeast Cakes: Principle. Unless yeast cells are destroyed by autolysis or heat, they yield only a small proportion of their thiamine content in the human gastrointestinal tract. Subjects in whom nutritional equilibrium has been established each receive, on a control day, 6 mg. of thiamine in pure solution and in divided doses after each meal. After an intervening period has elapsed, the same subjects receive 6 mg. of thiamine (determined by chemical analysis) in the form of live yeast cakes, similarly divided, after meals. The ratio of extra thiamine excretion in the test and control days is the measure of physiological availability of thiamine in the yeast product.

COMPOSITION OF BASAL DIET

Breakfast

1 orange	150 g.	2 butter squares	14 g.
2 slices of toast (enriched)	50 g.	1 glass of milk	230 g.

Luncheon

1 steak (lean)	150 g.	5 butter squares	35 g.
1 serving of fried potatoes	65 g.	1 glass of milk	230 g.
1 serving of carrots	60 g.	1 serving of apple pie	155 g.
1 serving of beets	70 g.	2 hard sugar candies	12 g.
2 slices of toast (enriched)	50 g.		

Supper

2 fried eggs	90 g.	1 glass of milk	230 g.
1 lettuce serving	25 g.	1 apple	150 g.
1 tomato serving	70 g.	1 banana	150 g.
2 slices of bread (enriched)	66 g.	2 hard sugar candies	12 g
4 butter squares	28 g.		

Analyses Conducted on Aliquots of the Composite Diet

Proximate Analysis	*Values Found*[451]	*Vitamin Content*	*Values Found*[451]
Total weight	2075 g.	Thiamine	1.34 mg.
Total solids	540 g.	Thiamine:Calorie ratio	0.5
Moisture	1535 g.	Thiamine:Nonfat calorie ratio	0.9
Protein	101 g.	Ascorbic acid	115 mg.
Fat (ether extract)	129 g.	Riboflavin	2.54 mg.
Ash	22 g.	Niacin	22.0 mg.
Crude fiber	6 g.		
Carbohydrate (by difference)	282 g.		
Caloric value	2710 Cal.		
Nonfat calories	1550 Cal.		

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[451] Expressed in terms of total food consumed in the three meals.

Procedure. A group of five subjects in good nutritional status, but not unduly saturated with respect to thiamine, is assigned for four days to a complete diet (as illustrated in the table above). The dietary schedule is as follows: a basal day followed immediately by a control (standard dose) day; then, after an interval of one to two weeks another basal day followed immediately by a test (assay dose) day. Just before the largest (noonday) meal of each basal day, the urine is voided and discarded. The urine collection is then begun (in a 2-liter brown glass bottle containing 20 ml. 10 per cent sulfuric acid as a preservative) this marking the beginning of the control (or test) period. On the control day, 2 mg. thiamine, dissolved in water or milk, is taken after each meal. The 24-hour urine is collected as described above. At least one week (preferably two) should elapse to provide for complete flushing of this control dose, during which no remaining collections are made and the subjects are free to eat adequate diets of their own selection.[452] The basal diet is then resumed and the urine collected for a 24-hour period. On the succeeding (test) day the same diet is consumed, but in place of the thiamine supplement of the control period each meal is supplemented with a suspension of yeast cake in milk or water furnishing the equivalent of 2 mg. of thiamine. (It is necessary, of course, to establish the thiamine content of the yeast under test by actual analysis rather than to assume the quantity stated to be present on the label.)

Analyses of the 24-hour urine collections made on the basal, control, and test days are performed according to the colorimetric method described on p. 1144.

CALCULATION. From the thiamine excretion of both the control and test days, subtract that of the preceding basal days. If the absolute increment of thiamine intake is not identical on the control and test days, express the increment in thiamine excretion as percentages of the respective supplementary doses. The extra excretion of thiamine following yeast dosage divided by that following dosage in the form of pure solution, multiplied by 100, gives the percentage physiological availability of the thiamine contained in the yeast.

The experiment may be so extended that each test period is allowed to run for 48 instead of 24 hours. In experiments reported by the authors of this method,[453] this was done in order to eliminate the possibility of a delayed rate of absorption of thiamine from live yeast cells as compared with pure solutions. That this does not occur is illustrated by the data in the table on p. 1285.

Effect of Dietary Thiaminase in Fish Products.[454] Chastek paralysis, an acute dietary disease of foxes, is caused by including 10 per cent or more of certain species of uncooked fish in the diet and may be cured or prevented by giving adequate amounts of thiamine.[455] Thiamine deficiency has been observed in cats fed a diet consisting exclusively of salt-water herring.[456] The enzymic nature of the antithiamine factor was suggested by the fact that concentrates possessed protein characteristics and reacted like a typical enzyme toward inhibitor substances.[457] The over-all reaction has been demonstrated to be the hydrolytic cleavage of the vitamin between the pyrimidine and thiazole rings.[458]

The antithiamine factor has been found in 15 out of 31 species of fresh-water fish

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[452] Pork meats and pharmaceutical vitamin preparations especially should be avoided.

[453] Hochberg, Melnick, and Oser: *J. Nutrition*, **30**, 201 (1945).

[454] Melnick, Hochberg, and Oser: *J. Nutrition*, **30**, 81 (1945).

[455] Green, Carlson, and Evans: *J. Nutrition*, **23**, 165 (1942).

[456] Smith and Proutt: *Proc. Soc. Exptl. Biol. Med.*, **56**, 1 (1944).

[457] Sealock and Goodland: *J. Am. Chem. Soc.*, **66**, 507 (1944).

[458] Krampitz and Woolley: *J. Biol. Chem.* **152**, 9 (1944).

RATE AND DEGREE OF URINARY EXCRETION OF THIAMINE FOLLOWING DOSAGE OF THE VITAMIN IN LIVE YEAST CAKES AND IN PURE SOLUTION

	Control Period			*Test Period*		
Subject	*Basal Excretion*	*After 5.76 mg. of Thiamine in Solution*[459]		*Basal Excretion*	*After 6 Live Yeast Cakes*[460]	
		1st 24 hrs.	*2nd 24 hrs.*		*1st 24 hrs.*	*2nd 24 hrs.*
	Mg. per day			*Mg. per day*		
D.M.	0.20	1.56	0.55	0.25	0.44	0.25
M.H.	0.20	1.68	0.59	0.24	0.56	0.37
E.M.	0.20	1.67	0.64	0.28	0.37	0.29
J.C.	0.26	1.78	0.57	0.23	0.70	0.40
H.H.	0.17	1.48	0.45	0.21	0.30	0.22
Average	0.21	1.63	0.56	0.24	0.47	0.31

$$\frac{0.47 - 0.24}{1.63 - 0.21} \times 100 = 16.2 \text{ per cent availability}$$

(as measured by first 24 hrs.' excretion).

$$\frac{(0.47 + 0.31) - 2(0.24)}{(1.63 + 0.56) - 2(0.21)} \times 100 = 17.0 \text{ per cent availability}$$

(as measured by 48 hrs.' excretion).

tested,[461] in clams,[462] in the Atlantic herring, in whiting, and in the Pacific mackerel; oysters are among the fish not containing this factor. In many parts of the world, fish are eaten raw or only slightly heated (e.g., smoked). The following experiment demonstrates the effect of the thiaminase in raw clams in subjects receiving thiamine as a dietary supplement, and the inhibition of this effect by heat-inactivation of the destructive enzyme. This experiment may be performed with certain other edible raw or lightly smoked fish.

Procedure. **Whip 100 g. raw clams in 400 ml. water in a Waring Blendor. Adjust 100 ml. of this suspension to pH 4.5, and boil under reflux for 20 minutes. Cool and readjust to original pH. Prepare an aqueous thiamine solution containing 250 μg. per ml. Set up three 100-ml. centrifuge tubes as follows: (1) 50 ml. water plus 1 ml. thiamine solution, (2) 50 ml. unheated clam suspension plus 1 ml. thiamine solution, (3) 50 ml. heated clam suspension plus 1 ml. thiamine solution.**

Store the suspensions for 6 hours at 37° C. with frequent agitation. Centrifuge. To 10 ml. of the clear supernatants, add 10 ml. of phenol-alcohol reagent and complete the colorimetric test for thiamine described on p. 1145.

For the *in vivo* availability study, choose five normal subjects with good dietary histories. Feed the basal diet shown on p. 1283, starting at noon, and collect 24-hour urine samples in 2-liter bottles containing 20 ml. 10 per cent

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[459] Taken as three 1.92-mg. doses of thiamine in milk, one after each meal.

[460] Containing 5.75 mg. of thiamine. Two cakes, containing 1.92 mg., suspended in milk and taken after each meal.

[461] Deutsch and Hasler: *Proc. Soc. Exptl. Biol. Med.*, **53**, 63 (1943).

[462] Woolley: Personal communication (1943).

sulfuric acid. On the second day, just after the midday meal, feed 5 mg. thiamine in 30 ml. aqueous solution and collect the second 24-hour samples.

Before starting the test period, allow two weeks to intervene during which the subjects return to their usual, adequate diets. The test period comprises four days. On the first three, feed the subjects the basal diet, but at the end of each meal, add a fresh, raw clam weighing approximately 35 g. (without the shell) which may be swallowed whole. At the beginning of the fourth day (after the midday meal) the subjects ingest 5 mg. thiamine in 30 ml. water followed by a clam. Continue the experiment till the end of the fourth day, feeding a clam with each meal as before. Collect 24-hour urine samples on all four days.

Analyze the urine samples for thiamine as described on p. 1144. Note the prompt and marked decrease in basal urinary excretion values when clams are ingested. Note also the effect of the clams on the test dose of the vitamin.

VI. STUDENT EXERCISES

1. *Demonstrations of Vitamins A, B_1, D, and G.* Divide at least six rats 21 to 28 days old into two groups so that there are twice as many rats in one group as in the other. Keep the rats in individual cages and follow the instructions for their care outlined on p. 1364 ff. Feed *ad lib.* one of the following diets to the larger group (experimental) and to the other group (preventive controls) feed the same diet plus one of the indicated supplements daily (except Sundays).

(*a*) *Vitamin A Deficient Diet* (*see p. 1262*): *Supplements.* One drop U.S.P. codliver oil daily; or one μg. of β-carotene in 0.1 ml. of cottonseed oil; or 3 drops of butterfat.

Weigh rats semiweekly and note changes in growth, vaginal smears (usually during the fourth week), condition of the eyes, and appearance of the fur. Compare the experimental group with the control group. Allow half of the experimental group to continue on the basal diet until they die, at which time perform autopsies, noting the presence of localized infections in the respiratory tract, tongue abscesses, renal calculi, etc. When growth has definitely ceased on the basal diet, begin to feed the remainder of the experimental group one drop of codliver oil (or other source of vitamin A). Record your observations.

(*b*) *Thiamine Deficient Diet* (*see p. 1147*): *Supplements.* 0.5 g. dried yeast; 3.0 g. whole wheat flour; 5 μg. of crystalline thiamine diluted in 100 mg. confectioners' sugar–cornstarch mixture (1:1).

Weigh rats semiweekly. Beginning with the third week note signs of polyneuritis. Spin the rats by rolling the tail between the hands. Convulsive seizures following this treatment are evidence of polyneuritis. When this is observed for two or three consecutive days, begin to feed half of the experimental group a thiamine-containing supplement. Allow the remainder of the experimental group to continue on the basal diet until death. Plot growth curves and compare with curative and preventive control groups.

(*c*) *Vitamin D Deficiency* (*see p. 1261*): *Supplements.* One drop of U.S.P. codliver oil; or 0.1 g. of irradiated yeast,[463] or one hour's exposure to sunlight (if the weather is sufficiently warm); or 15 minutes' exposure at a distance of 30 inches from a source of ultraviolet radiation. Feed the experimental and preventive control groups either of the rachitogenic diets described on p. 1261.

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[463] Irradiated yeast may be prepared by exposing a thin layer ($\frac{1}{8}$ inch) of dried brewers' yeast at a distance of 18 inches from a quartz mercury vapor or carbon arc lamp for 20 minutes, raking over the surface every 5 minutes.

Record body weights at semiweekly intervals, and after three weeks examine each rat for evidence of rickets. Note posture, gait, paralysis of hind legs, enlargement of knee joint, etc. Sacrifice one rat from each group, perform autopsy (note beading of rib cartilage) and line test of the tibia as described on p. 1265. If possible, take roentgenograms of at least one rat in each group (cf. Fig. 289). After definite, gross signs of rickets are observed in the experimental group (between three and four weeks), add a vitamin D supplement to the diet of half the group for a two-week curative period. Repeat above examinations for rickets. Compare all animals at autopsy. Dissect tibiae free from connective tissue, wrap in marked filter paper, extract for 24 hours with acetone in a Soxhlet extractor, dry in oven, and determine bone ash on "dry fat-free basis."

(*d*) *Riboflavin Deficient Diet* (*see pp. 1163-4*): *Supplements.* 3 ml. whole milk; or, 0.2 g. dried brewers' yeast; or, 10 μg. crystalline riboflavin.

Weigh rats semiweekly. When growth ceases in the experimental group, continue half of the group on the basal diet and add to the diet of the other half the same supplement fed the preventive control group. Plot growth curves over a total period of six to eight weeks. Note any signs of alopecia or dermatitis.

2. *Demonstration of Vitamin C Deficiency* (*Scurvy*). Divide six guinea pigs weighing about 280 to 300 g. into an experimental group of four, and a preventive control group of two. Feed both groups *ad lib.* one of the scorbutigenic diets described on p. 1239, supplementing the diet of the preventive controls daily with one of the following: 1.5 ml. of fresh orange juice; or, a fresh carrot or small potato; or, 0.5 ml. freshly prepared 0.1 per cent ascorbic acid. Liquid supplements should be pipetted directly into the mouth; the ascorbic acid solution may be sweetened with cane sugar.

Weigh guinea pigs semiweekly; beginning with the fourteenth day examine daily for signs of scurvy (see p. 1240) in the experimental group, particularly, sensitive joints, hemorrhagic gums, loose teeth, and characteristic posture. These should become pronounced during the fourth or fifth weeks. However, when definite symptoms are noted and growth has stopped, begin to feed half the experimental group the same antiscorbutic supplement which the preventive controls receive. Terminate the experiment after a six weeks' curative period. Autopsy each guinea pig either at death or at termination, comparing the negative controls with the preventive and curative groups. Examine especially for enlarged joints in ribs (beading) and leg bones, subcutaneous and intramuscular hemorrhages, loose, fragile teeth and soft hemorrhagic gums.

3. *Demonstration of Vitamin E.* Place two male (A and B) and two female (C and D) rats at weaning, on the vitamin E deficient diet described on p. 1274 and two males (E and F) and one female (G) on the same diet supplemented with a daily allotment of 3 drops of nonrancid wheat germ oil. At the age of about three months, mate these rats in the following sequence (removing the supplement-fed rats from the cage for their daily dose of wheat germ oil):

I. Male E with females C and D.
Allow mating to continue until each female shows a typical resorption. (The first impregnation may result in the birth of a litter.) (See IV.)

II. Male F with female G.
Allow mating to continue until two live litters are born and carried through the lactation period.

III. Males A and B with female G.
After one month, during which no pregnancy should result, kill male A, weigh the testicles and examine histologically. Compare with normal male E, of the same age.

IV. Begin feeding male B and female C, 3 drops of wheat germ oil daily at conclusion of previous matings. Mate F with C and D. Only C should produce a live litter while D should show resorption.

V. Male B with female G.
No live litter should result in spite of the proved fertility of female G. Record your observations and draw conclusions as to the effect of vitamin E deficiency on male and female fertility.

4. *Studies on the Planning of Experimental Diets.* With the aid of the data given in the various tables on pp. 1335 to 1363, and of other available reference books from which the calorific value, nitrogen content, vitamin content, etc., may be obtained, plan experimental diets as described below, using the following dietary constituents: Casein, egg albumin, gelatin, gluten, whole wheat, lean beef heart, dried milk, dried liver, starch, sugar, agar, lard, hydrogenated vegetable oil, butter, butterfat, codliver oil, dried yeast, orange juice, tomato juice, salt mixtures (see pp. 1262, 1374, and 1375) etc.

Plan diets which may be predicted to have the following effects, and hand in a report to your instructor explaining the reasons for your selections. Include in this report the calorific value, the protein content, and any other properties of the diet your instructor may suggest.

1. A diet that will *produce ophthalmia.*
2. A diet that will *promote growth but cause rickets.*
3. A diet that will *cure or prevent rickets.*
4. A diet that will *prevent growth but not cause rickets.*
5. A diet that will *restore growth following Diet 4.*
6. A diet that will *prevent normal reproduction.*
7. A diet that will *cause scurvy.*
8. A diet that will *cure or prevent scurvy.*
9. A diet that will *cause ketonuria.*
10. A diet that will *correct this ketonuria.*
11. A diet that will *cause polyneuritis.*
12. A diet that will *cure polyneuritis.*
13. A diet that will be *conducive to longevity and the birth and rearing of sturdy offspring.*
14. A diet that will *produce a dermatitis in rats.*
15. A diet that will *cure this dermatitis.*
16. A diet that will *cause anemia in rats.*
17. A diet that will *correct this anemia.*

CLINICAL VITAMIN DEFICIENCIES

SYLLABUS OF "STIGMAS, SYMPTOMS AND THERAPY" OF THE COUNCIL ON FOODS AND NUTRITION OF THE AMERICAN MEDICAL ASSOCIATION[464]

The stigmas and symptoms associated with deficiency of vitamin A, thiamine, riboflavin, niacin, ascorbic acid, vitamin D and vitamin K are listed in this syllabus together with a statement concerning treatment of each deficiency. Deficiencies of several vitamins, notably biotin, pyridoxine, pantothenic acid, and vitamin E, are not accompanied by stigmas which can be recognized at present.[465] The subject is in a stage of fluidity and development which probably will necessitate early revision or amplification. Particularly is this true of the diagnosis and treatment of deficiency of folic acid. This vitamin has been prepared in isolated form so recently that its consideration here is omitted. Not many of the stigmas listed are diagnostic of a vitamin deficiency in themselves, but the occurrence of several of these stigmas in association is at least presumptive evidence of some nutritional failure. Vitamin deficiencies commonly encountered in clinical practice are multiple. Scrutiny of the dietary history is indicated in cases in which several of the stigmas listed are present. Due attention should be paid to the well known fact that stresses such as pregnancy, exposure or disease may occasion the development of deficiency states when the diet otherwise might be considered adequate.

Treatment for a deficiency involves administration orally or, if need be, parenterally of large enough doses of the vitamin to be of therapeutic value and continuation of this treatment for long enough periods to assure a satisfactory therapeutic trial. However, since the diagnosis is necessarily presumptive in many instances, exclusive dependence on specific therapy is justified only infrequently, and basic to good treatment in all cases is a diet planned to be adequate nutritionally and assurance that the diet is eaten.[466] The diet is important for the education of the patient and as a means of dispensing factors heretofore not isolated which will be contained in the foods of such a diet. Likewise helpful in treatment because of its content of factors not as yet identified is some good source of the vitamin B complex as a whole. Products such as brewers' yeast or an extract of such yeast, wheat germ, extracts of cereal grasses or of rice bran, crude extract of liver or desiccated liver represent such sources. For a patient who cannot take foods or drugs orally or in whom absorption is poor, crude liver extract may be given intramuscularly or even on occasion it may be diluted with sterile isotonic solution of sodium chloride or dextrose and administered by vein.

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[464] Reproduced through the courtesy of the American Medical Association from *J. Am. Med. Assoc.*, **131**, 666 (1946).

[465] *Handbook of Nutrition*, A Symposium Prepared Under the Auspices of the Council on Foods and Nutrition of the American Medical Association, 1943.

[466] Jolliffe: *J. Am. Med. Assoc.*, **129**, 613 (1945).

Stigmas Suggesting Deficiency of Vitamin A

Xerosis of the conjunctiva:
Thickening with loss of transparency, so that only the more superficial vessels of the bulbar conjunctiva are clearly seen, associated with more or less yellow pigmentation, especially along the horizontal meridian of the eyeball; infrequently associated with small foamlike plaques called Bitot's spots.

Papular eruptions of pilosebaceous follicles:
A grater-like feel, which in early stages resembles gooseflesh but, when more fully developed, presents the picture of keratosis pilaris. The extensor surfaces of the arms and thighs and the flexor surfaces of the legs are primarily affected.

Xerosis or asteatosis of the skin:
Dryness, scaliness and crinkling, in extreme cases resembling alligator skin. In early stages the condition is associated with keratosis pilaris but it persists and extends after follicles have disappeared, the body hairs being broken and later lost. All parts of the body are involved, but the skin of the extremities, particularly of the legs, is more severely affected than the skin of the head and the trunk.

Follicular conjunctivitis:
Hypertrophy of the follicles, particularly of the lower eyelids.

Night blindness:
Conspicuous only in cases of advanced, severe deficiency.

Keratomalacia:
Thickening with subsequent ulceration and necrosis of the cornea: present only in most severe and advanced forms of deficiency.

Treatment of Vitamin A Deficiency

Early deficiency state:
25,000 U.S.P. units of vitamin A twice daily for two months or longer.

More chronic states:
25,000 U.S.P. units of vitamin A two to three times daily for a prolonged period.

Stigmas Suggesting Deficiency of Thiamine

Loss of strength of the quadriceps: disproportionate to loss of general strength, evidenced by difficulty in rising from the squatting position.

Loss of vibration sense: first of the toes and later of the malleoli and tibias.

Tenderness of the calves and hyperesthesia of the feet.

Diminution and loss of the achilles tendon and patellar reflexes first. Other tendon reflexes are lost in the later stages of the polyneuritis of "dry beriberi."

Edema of the shins, ankles and knee joints: found in "wet beriberi."

Treatment of Thiamine Deficiency

Acute deficiency state:
10 to 20 mg. or more of thiamine twice daily until relief of symptoms; this may be days or weeks.

Chronic deficiency state:
5 to 10 mg. or more of thiamine twice daily for a prolonged period.

Enlarged heart with dependent edema and elevated venous pressure: Poor response to rest and administration of digitalis unless thiamine is given. This is a late manifestation of severe deficiency (beriberi heart).

Papillary edema with retinal hemorrhages: associated with ophthalmoplegia and polyneuritis. The condition is a late manifestation of severe deficiency.

Stigmas Suggesting Deficiency of Riboflavin

Congestion of the limbic plexus:

Visible with a small hand lens or the +20 lens of the ophthalmoscope; invasion of the cornea by capillaries arising from this plexus (vascularization) requires a biomicroscope and slit lamp for detection.

Cheilosis:

Represented in chronic deficiency by excessive and irregular wrinkling, in acute deficiency by swelling and erasure of the normal wrinkling of the lips. Reddening, thinning, scaling, chapping of epithelium are associated.

Angular stomatitis:

Various combinations of erythema and open fissuring in the angles of the mouth with or without a white, moist maceration (perlèche); scars of healed fissures.

Dyssebacia:

An erythema overlaid with somewhat greasy, flaky accumulations resembling hoar frost, noted mostly in the alae nasi, canthi, pinnae, and other folds of the skin, accompanied in some cases by coarsening and elevation of the sebaceous follicles of the nose and cheeks, the latter also seen with deficiency of vitamin A.

Magenta tongue:

A purplish red coloring with moderate edema and flattening of filiform papillae; observed in more advanced deficiency.

Stigmas Suggesting Deficiency of Niacin

Edema of the tongue:

Shown by dental indentations.

Increased redness of the tongue:

Beefy red in chronic states; scarlet red in severe acute deficiency.

Congestion and hypertrophy of the papillae of the tongue, followed by fusion and atrophy:

In early stages the fungiform papillae are congested and hypertrophied. This is followed by

Treatment of Riboflavin Deficiency

Acute deficiency state:

5 mg. of riboflavin three times daily for weeks.

Chronic deficiency state:

3 to 5 mg. of riboflavin three times daily for a prolonged period.

Treatment of Niacin Deficiency

Acute deficiency state:

100 mg. or more of niacinamide twice daily for weeks:

Chronic state:

100 mg. of niacinamide twice daily over a prolonged period.

hypertrophy of the filiform papillae and later by their flattening. As they atrophy they fuse or mat together with multiple fissuring to give a cobblestone appearance and finally baldness. Vincent's infection of tongue and fauces, ulceration and pseudomembrane formation may or may not accompany these changes in the more advanced stages of this deficiency.

Dermatitis:

Erythema, rough scaling, with ulceration and formation of bullae, affecting primarily areas of the skin exposed to light; namely, wrists, ankles, neck, and face; observed only in severe deficiency (pellagra) and then frequently associated with diarrhea and dementia.

Encephalopathy:

Clouding of consciousness, cogwheel rigidity, and grasping, sucking reflexes observed in acute, severe deficiency.

Stigmas Suggesting Deficiency of Ascorbic Acid

Redness, edema, tenderness, and bleeding on pressure of the gums:

Observed in acute or subacute deficiency of moderate severity, sometimes with, but usually without, other signs of ascorbic acid deficiency.

Thickening and increased firmness of the gums:

With recession and exposure of the base of the teeth, including recession of interdental papillae; observed in chronic deficiency.

Retraction of the gums:

Leaving pockets between gum and tooth, secondary infection and resulting pyorrhea; observed in chronic deficiency.

Loosening and shedding of the teeth.

Increased capillary fragility:

Manifested by petechial hemorrhages of the skin, especially in the tourniquet test; observed in more severe acute and subacute deficiency. Easy bruising, spontaneous ecchymosis of the skin, idiopathic hemorrhage into joints and slow healing of wounds, observed in severe acute and subacute deficiency.

Stigmas Suggesting Deficiency of Vitamin D

Deformities of the skeleton:

Bowed legs, malformation of the chest (funnel breast) and defects of the teeth may be residues of early rickets which is usually no longer active or susceptible to treatment; enlargement of the wrists, elbows, knees, ankles and costochondral

Treatment of Ascorbic Acid Deficiency

Acute or subacute deficiency state:

100 mg. or more of ascorbic acid daily for weeks.

Chronic deficiency state:

100 mg. of ascorbic acid three times daily over a prolonged period.

Treatment of Vitamin D Deficiency

In infants:

1,500 to 2,500 U.S.P. units of vitamin D daily, continued for several months (double this amount for premature infants).

junctions (beading, rachitic rosary) and bulging forehead (cranial bosses) which respond to treatment are found in infancy and in the rare example of late rickets.

Stigmas Suggesting Deficiency of Vitamin K

A tendency to bleeding:

Particularly from minor wounds, related to abnormal lengthening of the prothrombin time, developing spontaneously in newborn infants; observed in adults after treatment with dicoumarol or large doses of salicylates; in advanced disease of the liver with poor excretion of bile, and in disease of the intestine, such as sprue in which vitamin absorption is disturbed.

Treatment of Vitamin K Deficiency

In adults:

1 mg. of vitamin K two to three times daily with or without bile (1 g. of desiccated bile or bile salts).

In newborn babies:

1 mg. of synthetic vitamin K intramuscularly daily in oil solution for several days.

Deficiency Symptoms

A diagnosis of vitamin deficiency only rarely can be based on symptoms or less significant abnormalities than those which have been listed thus far. However, such symptoms and abnormalities frequently accompany the more specific lesions of deficiency.

Symptoms commonly observed with deficiency of thiamine, also less conspicuously in deficiency of other vitamins, include apathy, lethargy, increased emotional irritability, hypersensitivity to noise and painful stimuli, headache, vague fears, confusion of thought, uncertainty of memory, asthenia, loss of manual dexterity, insomnia, heart consciousness, paresthesia, anorexia, nausea, flatulence, epigastric pain, constipation.

Photophobia, burning of the eyes, lacrimation and eyestrain not relieved by glasses are encountered in deficiency of riboflavin.

Other abnormalities unrelated to deficiency of any single vitamin but commonly observed in persons who are malnourished are dry, brittle, lack-luster, rebellious, so-called staring head hair, a loss of sleekness analogous to the rough coat of malnourished animals, blepharitis, spider-like telangiectasis of the face, seborrhea of the face, patchy pigmentation of the face, especially suborbital and circumoral, sinus arrhythmia, bradycardia, tachycardia, low blood pressure, loss of tone of muscles and anemia.

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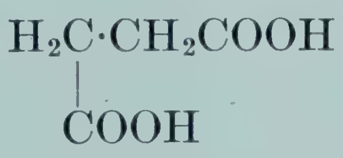
36

Metabolic Antagonists and Antibiotics

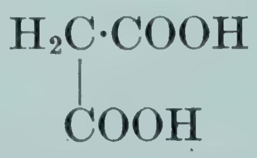
Few, if any, enzymes or enzyme systems have absolute specificity for a single substrate. For example, the hippuricase of Taka-diastase will not only catalyze the hydrolysis of hippuric acid, but also that of *m*- and *p*-chloro-, bromo-, iodo-, nitro-, and methyl-hippuric acids. The corresponding ortho substituted acids unite with the enzyme, but hydrolysis of the peptide linkage does not occur and the enzyme is rendered inactive.[1] There are many similar instances in which compounds other than the usual substrate will combine with an enzyme, but do not react further. Such blocking compounds are usually structurally similar to the normal substrate. The enzyme so blocked is unable to combine with its usual substrate, and the reaction catalyzed by the enzyme is slowed or stopped according to the proportion of total enzyme molecules thus blocked.

Perhaps the simplest example of inhibition related to structural analogy is found in the case of the enzyme succinic dehydrogenase. This enzyme catalyzes the oxidation of succinic acid to fumaric acid in the presence of a hydrogen acceptor (see p. 318). The action of succinic dehydrogenase on succinic acid is almost completely inhibited by the presence of malonic acid.[2] The relation between these two compounds is evident from a comparison of their structures:



$$\begin{array}{l} H_2C\cdot CH_2COOH \\ \quad | \\ \quad COOH \end{array}$$

Succinic acid



$$\begin{array}{l} H_2C\cdot COOH \\ \quad | \\ \quad COOH \end{array}$$

Malonic acid

The inhibition by malonic acid is reversible, that is, it may be overcome by the addition of a sufficient amount of succinic acid, in which case the enzyme behaves as though no inhibitor were present. This reversible relation between succinic and malonic acids, together with the obvious fact that these two substances cannot react with each other, implies that both types of molecule are competing for a reactive group on the enzyme molecule itself. This is therefore an example of *competitive inhibition*. Since the rate of formation of a particular enzyme-substrate complex depends upon the frequency of collisions of substrate molecules with the enzyme surface, which in turn depends upon the concentration of substrate, the extent to which malonic acid will inactivate succinic dehydrogenase depends upon the *relative* concentrations of succinic and malonic acids. This relationship defines competitive as opposed to noncompetitive inhibition. In any competitive inhibition—

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[1] Ellis and Walker: *J. Biol. Chem.*, **142**, 291 (1942).

[2] Quastel and Wooldridge: *Biochem. J.*, **21**, 1224 (1927).

$$E + S \rightleftarrows ES \rightarrow E + P$$
$$E + I \rightleftarrows EI$$

where E is the enzyme involved, S is its normal substrate, I is the competitive inhibitor, usually a structural analog, and P is the product. By the mass-action law, the dissociation constant of the enzyme-substrate complex is K_s

$$K_s = \frac{[E][S]}{[ES]}$$

and the dissociation constant of the enzyme-inhibitor complex is K_I.

$$K_I = \frac{[E][I]}{[EI]}$$

Dividing K_I by K_S, we can get an inhibition constant, K.

$$K = \frac{[I][ES]}{[S][EI]}$$

At 50 per cent inhibition

$$[ES] = [EI]$$

and

$$K = \frac{[I]}{[S]}$$

Thus, the effectiveness of a competitive inhibitor depends upon the *ratio* of concentrations of inhibitor and normal substrate, and not upon their absolute amounts. The relationship of competition applies to a number of well-known enzyme inhibitions, including the inhibition of saccharase by fructose, and of xanthine oxidase by adenine.

In considering enzymes which have several substrates (e.g. amine oxidase) it is not always possible to make a sharp distinction between substrates and competitive inhibitors. One substrate may be considered to be competing with the other substrates.

Another example of what is believed by many to be an instance of competitive inhibition is found in the action of the sulfonamides on bacteria. In studying the action of sulfanilamide on certain bacteria, Woods[3] found that the growth-inhibiting effect of this compound could be reversed by a substance present in yeast extracts. This substance was believed to be structurally similar to sulfanilamide, so a number of structural analogs were tested for their antisulfanilamide activity. Of these, *p*-aminobenzoic acid (PABA) proved to be remarkably effective; the structural similarity of this compound to sulfanilamide is evident from a comparison of their structures:

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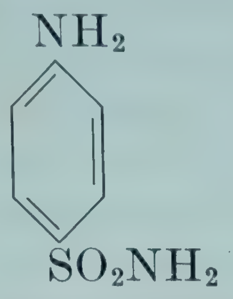
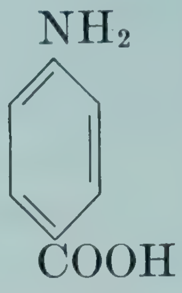
[3] Woods: *Brit. J. Exptl. Path.*, **21**, 74 (1940).


Sulfanilamide


***p*-Aminobenzoic acid**

On the basis of his results, Woods suggested that *p*-aminobenzoic acid should prove to be an essential growth factor for bacteria, and this was later shown to be the case.

Lampen and Jones[4] related the need of certain organisms for *p*-aminobenzoic acid to its utilization in the formation of folic acid (see p. 1195). Sulfonamides competitively inhibit enzymatic reactions whereby *p*-aminobenzoic acid is built into the folic acid molecule. Most organisms, probably all organisms, require folic acid. Organisms that require their folic acid ready-made are not hampered by sulfonamides, which interfere with folic acid formation; but growth of such organisms is inhibited by certain structural analogs of folic acid itself, such as aminopterin.[5] Those organisms which require *p*-aminobenzoic acid for the synthesis of folic acid within the organism are subject to sulfonamide inhibition, which can be released competitively by *p*-aminobenzoic acid or noncompetitively by folic acid. If enough folic acid is present to meet the growth requirement, no inhibition of growth of *Streptococcus faecalis* will be brought about by sulfonamides. Still other organisms exist, including many common pathogens, which are sensitive to sulfonamides, but are not released from growth inhibition by folic acid. In such instances the essential substance synthesized from *p*-aminobenzoic acid may be a derivative of folic acid (such as the citrovorum factor, p. 1201), and folic acid itself may not be an intermediate in the formation of the essential substance. Alternatively, in some organisms *p*-aminobenzoic acid may have important functions other than folic acid synthesis. Each microbial species must be evaluated for its requirement of folic acid and related substances. As an example of a notable difference, *Streptococcus faecalis* requires folic acid and synthesizes it from *p*-aminobenzoic acid, whereas *Lactobacillus arabinosus* breaks down added folic acid and utilizes the components.[6]

Fluoroacetate[7] is a metabolic analog of acetate. Like acetate, fluoroacetate can form an active 2-carbon fragment which proceeds to form a fluorotricarboxylic acid. This product appears to be the actual antimetabolite, which blocks the normal conversion of the acids of the citric series into α-ketoglutaric acid. The result is accumulation of citrate.

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[4] Lampen and Jones: *J. Biol. Chem.*, **166**, 435 (1946).

[5] Aminopterin (4-aminopteroylglutamic acid) is the structural analog most antagonistic to folic acid and diminishes the utilization of formate in purine and pyrimidine synthesis. Aminopterin and other folic acid antagonists have been used in the treatment of leukemia. For a full discussion of folic acid antagonists, see Petering: *Physiol. Revs.*, **32**, 197 (1952).

[6] Koft, Sevag, and Steers: *J. Biol. Chem.*, **185**, 9 (1950).

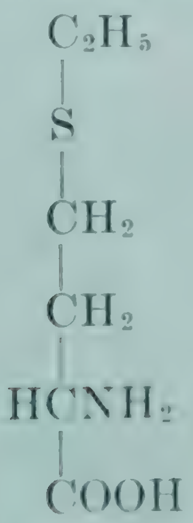
[7] Peters: *Brit. Med. J.*, Nov. 29, **1952**, 1165. Do not confuse fluoroacetate with iodoacetate which is not an antimetabolite. Iodoacetate is a nonspecific inactivator of sulfhydryl groups, the integrity of which is necessary for the action of numerous enzymes.

There is another type of inhibition of biological systems and its reversal which has not always been recognized as being somewhat different from the type described here. This is the condition in which an inhibition of enzymatic or cellular activity by one substance is reversed—i.e., the activity is restored—by the addition of a second substance which is capable of reacting directly with the inhibitor. For example, many compounds are known which react with the sulfhydryl group (—SH). Certain of these compounds will inactivate biological systems, and the activity may be regenerated by adding an excess of a second substance which contains sulfhydryl groups, such as cysteine or mercaptoacetic acid. It is to be noted, however, that the substance which overcomes the inhibition is not necessarily a constituent of the biological system being acted upon by the inhibitor, and the reversal of inhibition is essentially one of neutralizing the activity of the inhibitor by reacting directly with it. While this type of study may suggest the kind of compounds or active groups which function in biological systems, it is clearly different from metabolic antagonism as described here.

In the following pages examples are given of what appears to be metabolic antagonism between structurally related compounds. It must be recognized that in practically all instances the precise mode of action of even the normal metabolite is relatively unknown; the action of the antagonist, therefore, can only be a subject for speculation. There is, nevertheless, a fundamental similarity in action among all of the substances to be described, in that their inclusion in a normally functioning biological system leads to signs of a deficiency which resembles that produced by a lack of the normal metabolite concerned, and which can be overcome by the presence of sufficient amount of the latter. The substances to be discussed include structural analogs of the naturally occurring amino acids, vitamins, and hormones.

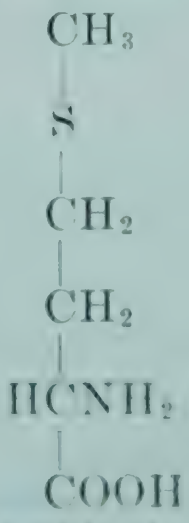
ANALOGS OF AMINO ACIDS

Methionine and Ethionine. In 1938 Dyer[8] reported that the S-ethyl analog of methionine, called ethionine, appeared to be toxic to rats on a low-cystine, low-methionine diet, whereas in the presence of sufficient methionine loss of weight and death did not occur.



$$\begin{array}{c} C_2H_5 \\ | \\ S \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ HCNH_2 \\ | \\ COOH \end{array}$$

Ethionine



$$\begin{array}{c} CH_3 \\ | \\ S \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ HCNH_2 \\ | \\ COOH \end{array}$$

Methionine

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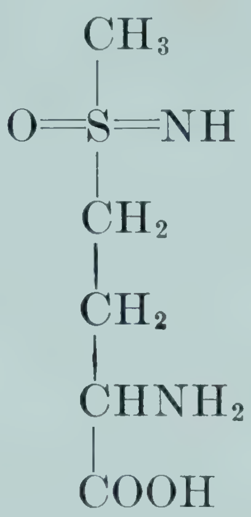
[8] Dyer: *J. Biol. Chem.*, **124**, 519 (1938).

Subsequent work has shown that ethionine blocks several vital functions of methionine: (*a*) transmethylation, (*b*) incorporation of methionine and other amino acids into protein, (*c*) formation of cystine, and (*d*) lipotropic action. The antibacterial action of sulfonamides is partially antagonized by methionine, but is augmented by ethionine. The sulfonamides compete with *p*-aminobenzoic acid in its function as part of a coenzyme related to the methylation of homocysteine.

The experimental results of Dyer represent probably the first authentic demonstration of metabolic antagonism involving amino acids, in terms of the concept as it has been presented in the preceding pages.

Another example of an antimetabolite to methionine is found in the compound known as methionine sulfoximine:



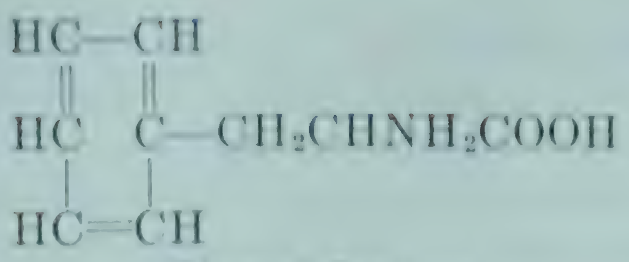
$$\begin{array}{c} CH_3 \\ | \\ O{=}S{=}NH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array}$$

Methionine sulfoximine

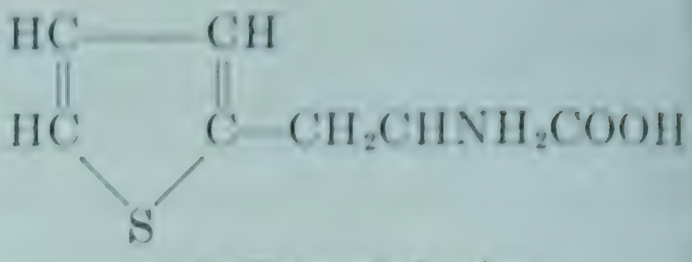
This substance was first isolated from the hydrolyzate of the protein of wheat flour which had been oxidized (matured) by means of nitrogen trichloride (Agene). Such flour is toxic to dogs, producing the disease known as canine hysteria or running fits. The toxic principle was isolated with the aid of chromatographic techniques and was proved to be a derivative of the amino acid methionine, produced by the action of the nitrogen trichloride on the methionine of the wheat-flour protein. The compound has been synthesized and given the name methionine sulfoximine because of its structure. The compound shows a high degree of species specificity, being much more toxic to dogs than to other laboratory animals, but investigations have revealed no evidence of any effect of the treated flour on human beings. However, nitrogen trichloride is no longer permitted in the United States for maturing flour.

The toxic effect of methionine sulfoximine on dogs can be readily overcome by the addition of methionine to the diet. The growth of certain microorganisms can be inhibited by low concentrations of methionine sulfoximine, and this effect is reversed by methionine, glutamine, and to some extent by methionine sulfoxide (but not by methionine sulfone or glutamic acid). Thus methionine sulfoximine appears to be a true antimetabolite but the mechanism of this action is still obscure.

Phenylalanine and Thienylalanine. The synthetic compound β-2-thienylalanine is similar in structure to the amino acid phenylalanine except that a —CH=CH— group in the benzene ring of phenylalanine is replaced by —S— in thienylalanine:



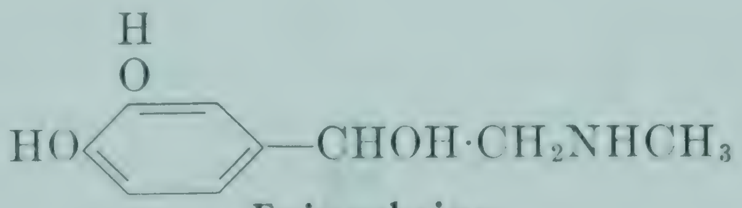
Phenylalanine



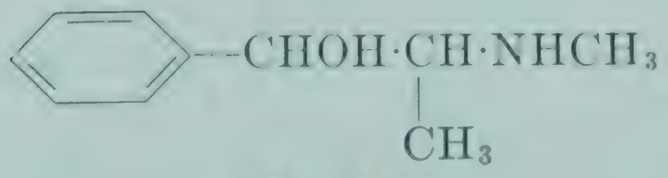
β-2-Thienylalanine

The growth of certain yeasts and bacteria is inhibited by thienylalanine and the inhibition is overcome by phenylalanine. The growth inhibition of *E. coli* caused by 100 parts of thienylalanine was 50 per cent nullified by 5 parts of phenylalanine, but tryptophan was almost as effective as phenylalanine. Inhibition of yeast growth by thienylalanine was overcome by certain other amino acids in 10 to 30 times the concentration of phenylalanine required. The tubercle bacillus, which is resistant to most growth inhibitors, is susceptible to thienylalanine, as is vaccinia virus, although in the latter case action on the host cell can not be ruled out. Studies with β-3-thienylalanine have indicated that it is a somewhat more effective phenylalanine antagonist than its β-2 isomer, whereas β-2-furylalanine is less effective. Inhibition of weight gain in young or protein-depleted rats can be produced by phenylalanine antagonists, and reversed by phenylalanine but not by tyrosine. Halogenated phenylalanines and phenylserine have been shown to be competitive antagonists to phenylalanine in several microbial species. A very important noncompetitive antagonist to phenylalanine is chloramphenicol, one of the broad-spectrum antibiotics (see p. 1317).

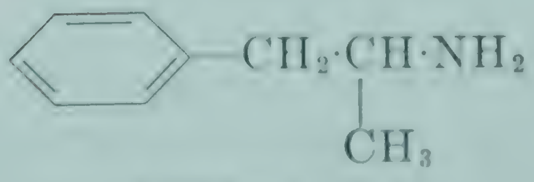
The enzymes which catalyze the oxidation of phenylalanine to tyrosine, and through further steps to melanin, may be blocked by certain analogs. Thienylalanine will block the first step in *E. coli;* the oxidation of tyrosine by bacterial and mammalian tyrosinases is blocked by *N*-acetyl, *N*-formyl-, or 3-fluorotyrosine. Epinephrine and norepinephrine are inactivated in the body by several enzymatic mechanisms. One of these, amine oxidase, is competitively blocked by *ephedrine*, *amphetamine*, and other related compounds. This blockage of epinephrine inactivation is not a complete explanation of the pharmacological actions of these sympathicomimetic drugs.



Epinephrine

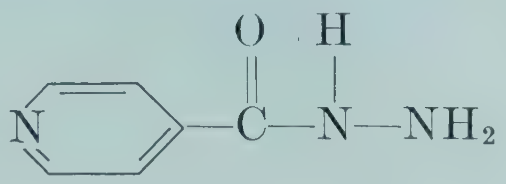


Ephedrine

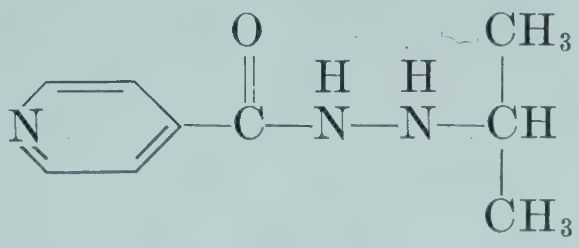


Amphetamine

Isonicotinic acid hydrazide is inhibitory to amine oxidase, and 1-isonicotinyl-2-isopropylhydrazine is about 10 times as effective.



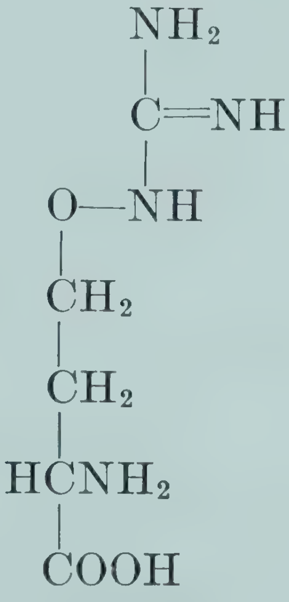
Isonicotinic acid hydrazide



1-Isonicotinyl-2-isopropylhydrazine

These two compounds, like other basic antitubercular drugs and antibiotics, inhibit the diamine oxidase of mycobacteria.[9] Against mammalian diamine oxidase, the antibiotics streptomycin and neomycin have little effect, but the two hydrazides inhibit both the bacterial and the mammalian enzyme.

Other Amino Acids. The amino acid L-canavanine, which occurs in jack beans, is a competitive antagonist to arginine and inhibits growth of *E. coli*, of certain strains of *Neurospora*, and of lactic acid bacteria. Arginase, however, will split urea from canavanine. Citrulline and ornithine will release organisms capable of utilizing these substances from canavanine inhibition.



$H_2N-C(=NH)-NH-O-CH_2-CH_2-HCNH_2-COOH$

Canavanine

One general method of modifying amino acids consists in replacing the carboxyl group by the sulfonic acid group, as in the case of sulfanilamide and *p*-aminobenzoic acid (p. 1299). These aminosulfonic acids prove to be growth-inhibitory to microorganisms but the inhibition is apparently nonspecific, since it may be reversed by aminocarboxylic acids not structurally related to the inhibiting compound. McIlwain[10] interprets this as indicating that structural analogy alone is not the sole consideration in explaining the action of metabolic antagonists. Indoleacrylic acid, the deaminated α, β unsaturated analog of tryptophan, has been reported to inhibit the growth of *E. coli*, and this inhibition is overcome by tryptophan, but apparently not competitively.

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[9] Owen, Karlson, and Zeller: *J. Bact.*, **62**, 53 (1951).

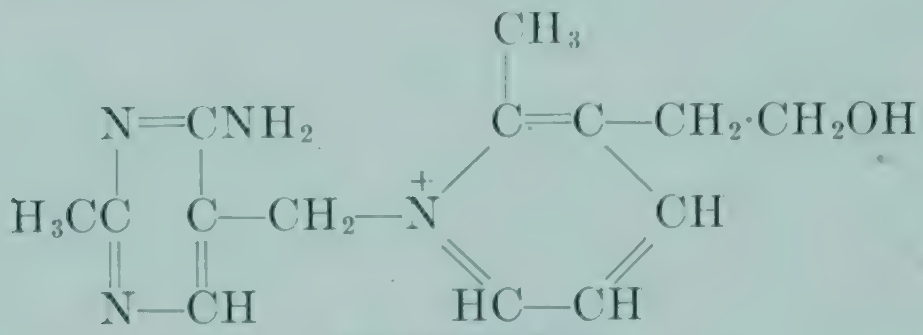
[10] McIlwain: *Brit. J. Exptl. Path.*, **22**, 148 (1941).

The D and L forms of an amino acid would appear to be competitive analogs in those instances where only one of the two forms is metabolized by an organism. While there are numerous instances demonstrating the lack of availability of one of the two enantiomorphs of an amino acid for growth, there are relatively few examples where competitive inhibition has been demonstrated.

There are numerous instances in which naturally occurring amino acids compete with each other; for example, arginine competes with lysine in a mutant strain of *Neurospora*. This and other examples have been tabulated by Martin. The possibility that competition between naturally occurring substances plays a part in regulating metabolic processes can not be overlooked. Martin postulates, as the "relativity theory" of the biological world, that natural metabolite antagonisms are fundamental to life by virtue of stabilizing relative concentrations of available nutrilites. In pharmacology and therapeutics, numerous applications are made of the theory of metabolite antagonism. In relatively few instances can competitive relationship be demonstrated; more often, the action is noncompetitive or irreversible.

ANALOGS OF VITAMINS

Thiamine and Pyrithiamine. Pyrithiamine is the pyridine analog of thiamine, obtained by replacing the —S— group in the thiazole ring of thiamine by —CH═CH—; the structural relationship is thus similar to that between thienylalanine and phenylalanine.



Pyrithiamine

Pyrithiamine was found by Robbins[11] to be inhibitory to the growth of certain microorganisms which required an exogenous supply of thiamine for growth, and Woolley and White[12] showed that the feeding of pyrithiamine to mice produced a severe deficiency which resembled that of thiamine deficiency and which could be overcome by the administration of thiamine; one molecule of thiamine was required for approximately 40 molecules of pyrithiamine. This is one of the earliest instances of a deficiency disease produced in animals by the administration of a structural analog of a vitamin.

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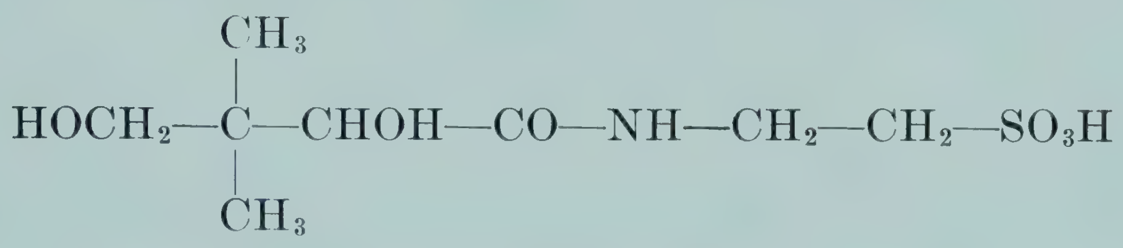
[11] Robbins: *Proc. Nat. Acad. Sci.*, **27**, 419 (1941).

[12] Woolley and White: *J. Biol. Chem.*, **149**, 285 (1943).

Studies on the inhibition of growth of microorganisms by pyrithiamine have revealed that the substance is inhibitory only in those instances where the organism requires an external supply of thiamine. If the organism is capable of synthesizing thiamine from the constituents of the medium, pyrithiamine is not inhibitory to growth. This emphasizes the point already made that structural analogy per se is not necessarily the sole factor in determining whether competitive inhibition will occur, particularly in the complex metabolic processes of living protoplasm. One interesting example has been presented of a strain of yeast which became resistant to the inhibitory effect of pyrithiamine apparently by the development of a system which permitted liberation of the pyrimidine portion of the molecule and its subsequent utilization in the synthesis of thiamine itself.[13]

Certain other structural analogs of thiamine have likewise been shown to be antagonistic to thiamine; for example, oxythiamine, in which the NH_2 of thiamine is replaced by OH. The thiamine molecule is particularly susceptible to such chemical modification, consisting as it does of two reactive portions, the pyrimidine moiety and the thiazole portion.

Pantothenic Acid and Pantoyltaurine. The replacement of the carboxyl group of pantothenic acid by the sulfonic acid group[14] produces a substance known as pantoyltaurine (or thiopanic acid):



$$HOCH_2—\overset{\displaystyle CH_3}{\overset{|}{\underset{\displaystyle CH_3}{\underset{|}{C}}}}—CHOH—CO—NH—CH_2—CH_2—SO_3H$$

Pantoyltaurine

This substance and related compounds, e.g. the amide, have been shown to be antagonistic to pantothenic acid for a variety of microorganisms; growth inhibition caused by pantoyltaurine can be overcome in most instances by pantothenic acid, and the inhibition appears to be truly competitive in nature. As with thiamine and pyrithiamine, pantoyltaurine is not inhibitory to the growth of those microorganisms which can synthesize the pantothenic acid they need.

Pantoyltaurine has not as yet been shown to produce unequivocal signs of a pantothenic acid deficiency in animals, probably because of the relatively large amounts which would be required to compete successfully with the pantothenic acid present in animal tissues. Some success has been obtained in the use of pantoyltaurine as a chemotherapeutic agent in rats infected with an organism susceptible to inhibition by pantoyltaurine, but here again the doses required were so large as to indicate little practical application of this fact. The possibility remains open, however, that further studies along these lines may produce more effective substances. As with thiamine, the presence of two chemically distinct portions in the pantothenic acid molecule has permitted the synthesis of a variety of structural analogs other than pantoyltaurine, and certain of these appear to be effective in inhibiting the growth of microorganisms in a manner similar to that described for pantoyltaurine.

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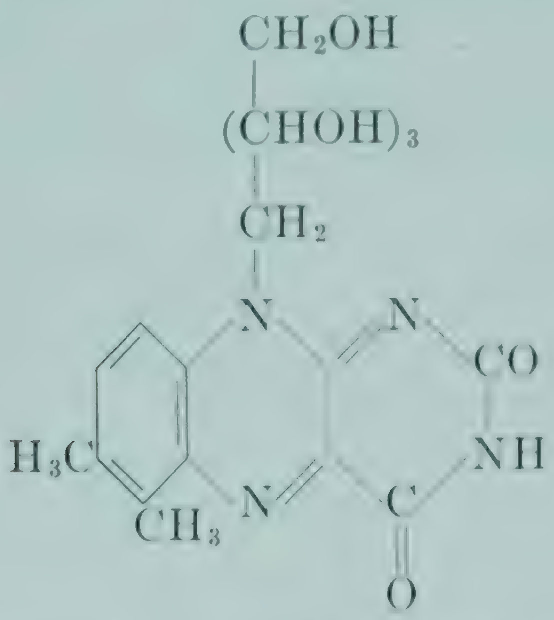
[13] Woolley: *Proc. Soc. Exptl. Biol. Med.*, **55**, 179 (1944).

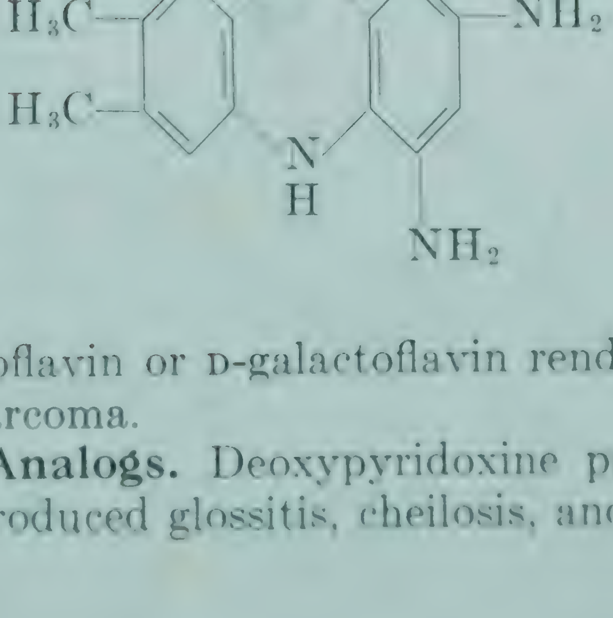
[14] Snell: *J. Biol. Chem.*, **139**, 975 (1944).

Riboflavin and Analogs. Various structural analogs of riboflavin have been synthesized which appear to be metabolic antagonists of the naturally occurring vitamin. One of these is isoriboflavin, in which the two methyl groups are in the 5,6 positions instead of in the 6,7 positions as in the naturally occurring vitamin:



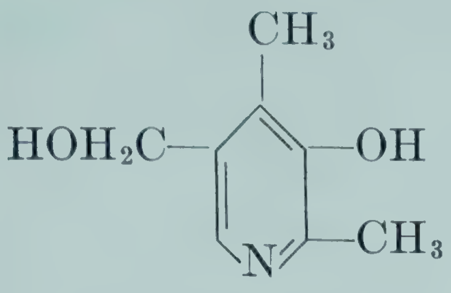
5,6-Dimethyl-9-(D-1′-ribityl)-isoalloxazine
(Isoriboflavin)

The onset of the symptoms of riboflavin deficiency can be hastened in rats already on a suboptimal diet by the feeding of isoriboflavin, but this substance does not function as a riboflavin antagonist in any bacterial species so far studied. Antagonists similarly effective in animal experiments have been prepared by substituting sugars other than ribose in the 9-position. Effective both in animals and bacteria is 2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine:

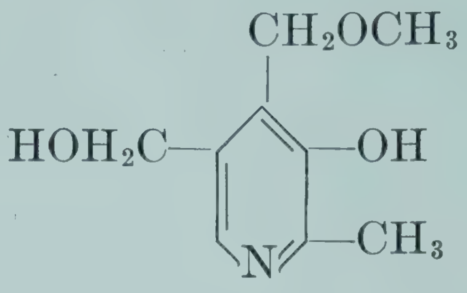


Large doses of isoriboflavin or D-galactoflavin render mice refractory to implants of lymphosarcoma.

Pyridoxine and Analogs. Deoxypyridoxine produces acrodynia in the mouse and has produced glossitis, cheilosis, and seborrhea in human subjects, corrected promptly by pyridoxine. Methoxypyridoxine is an effective antagonist to pyridoxine in chicks but is much less active in rats and is inactive in the mouse, which can form pyridoxine from methoxypyridoxine. A number of other analogs are antagonistic to pyridoxine when tested with yeast.

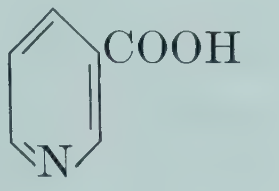


Deoxypyridoxine

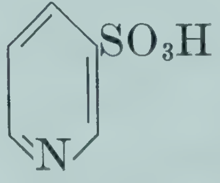


Methoxypyridoxine

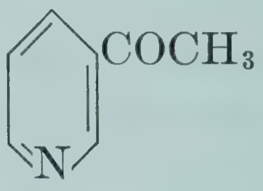
Nicotinic Acid and Analogs. Analogs of nicotinic acid which appear to act as competitive antagonists of the latter have been obtained by replacing the carboxyl group of nicotinic acid with either the sulfonic acid group or the acetyl group:



Nicotinic acid



Pyridine-3-sulfonic acid



3-Acetylpyridine

The analogous amides have also been studied. The sulfonic acid analog produces inhibition of growth of certain microorganisms which can be overcome by nicotinic acid, but is apparently without effect on animals. As with certain other inhibitors already discussed, pyridine sulfonic acid is without effect on those species of microorganisms which can synthesize nicotinic acid, again suggesting that cell penetration or local concentration may be as important as simple structural analogy in explaining the action of metabolic antagonists. The acetylpyridine analog of nicotinic acid brings about reversible signs of nicotinic acid deficiency in mice and dogs, but is without effect on those microorganisms which have been studied.

Ascorbic Acid and Glucoascorbic Acid. It was shown by Woolley and Krampitz[15] in 1943 that a synthetic compound called glucoascorbic acid produced scurvylike symptoms when fed to mice on a highly purified diet. These symptoms could not be cured when ascorbic acid was also included in the diet, but disappeared when the glucoascorbic acid was omitted. It will be recalled that mice do not require ascorbic acid in the diet, apparently being capable of synthesizing the amount they need. In the case of the guinea pig, however, which develops scurvy on diets free from ascorbic acid, Woolley has reported that a disease which is not exactly like scurvy but which has some similarities to it is produced on diets containing glucoascorbic acid, and ascorbic acid counteracts this effect. The structural relationship between ascorbic acid and glucoascorbic acid is evident from the following:

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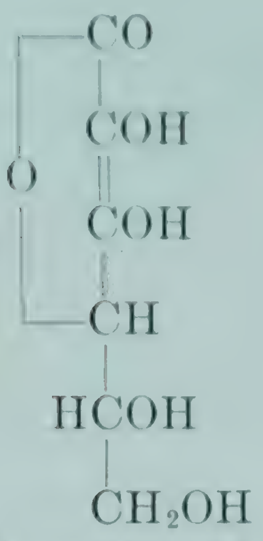
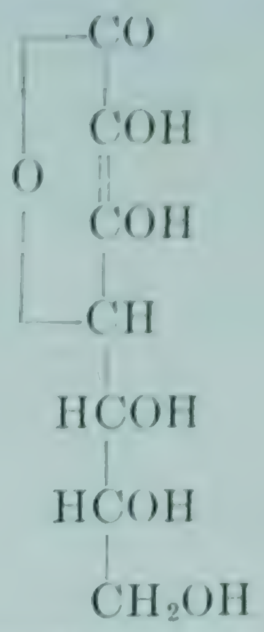
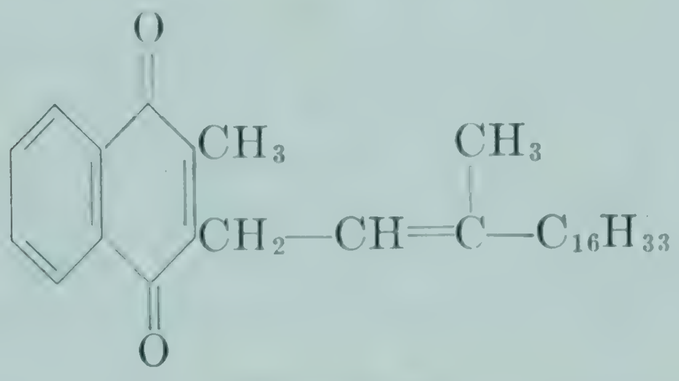
[15] Woolley and Krampitz: *J. Exptl. Med.*, 78, 333 (1943).

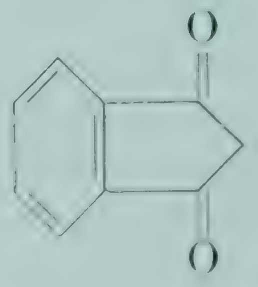

Ascorbic acid

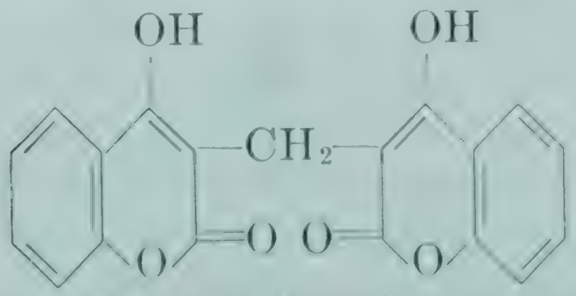

Glucoascorbic acid

It appears likely that the relation between ascorbic acid and glucoascorbic acid is one of metabolic antagonism, although the evidence is more limited than for most other examples of this phenomenon.

Vitamin K and Analogs. A hemorrhagic disease occurs in cattle from eating spoiled sweet-clover hay. The substance responsible is *dicoumarol* (see formula), which is an antagonist of the K vitamins, interfering with their utilization in the synthesis of prothrombin. Dicoumarol and several related compounds have been used extensively for the prevention of thrombosis. Derivatives of 1,3-indandione have been investigated for anticoagulant activity, and successful clinical use has been reported for the phenyl and the diphenylacetyl derivatives.


Vitamin K_1


1,3-Indandione


3,3′-methylene-bis(4-hydroxycoumarin)
(Dicoumarol)

Biotin and Analogs. The structural relation to biotin of some analogs or biotin which have been shown by Dittmer and du Vigneaud[16] to have antibiotin activity is as follows:

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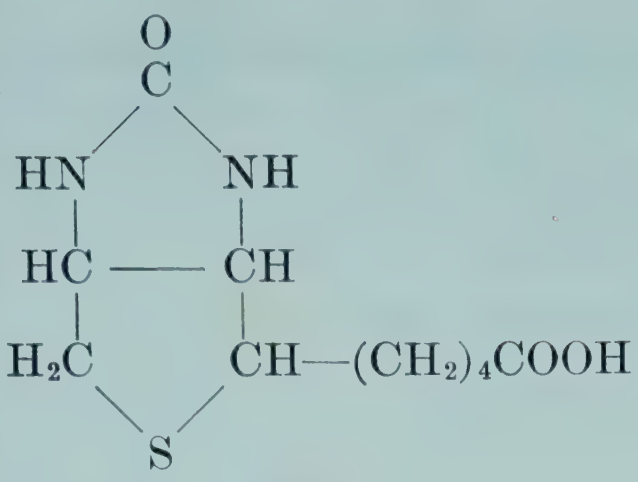
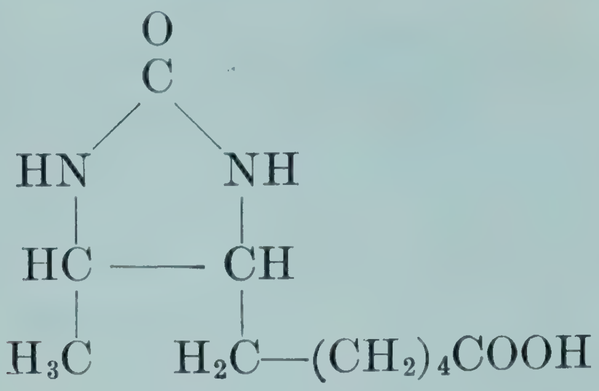
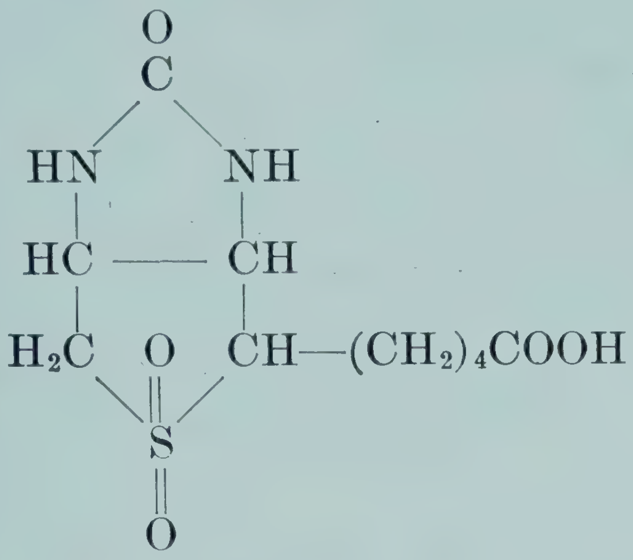
[16] Dittmer and du Vigneaud: *Science*, **100**, 129 (1944).



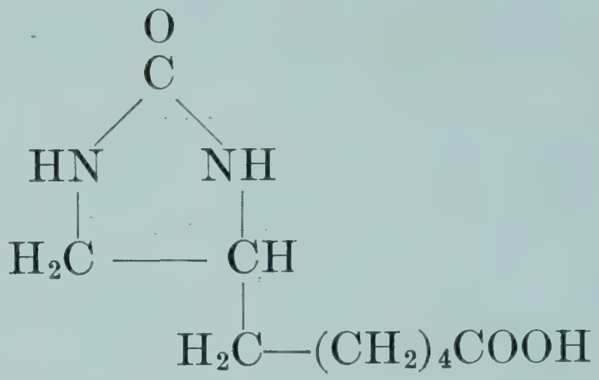
Biotin



Dethiobiotin



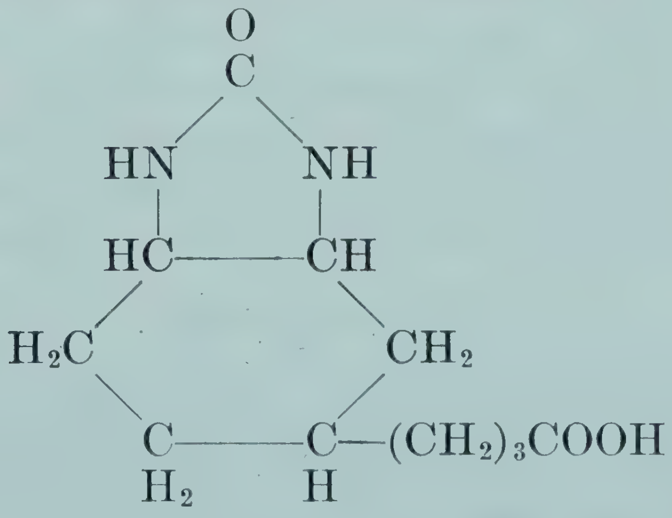
Biotin sulfone



Imidazolidone caproic acid

Evidence for competition between biotin and its structural analogs has been obtained largely by the use of yeast and bacteria. Inhibition of growth of microorganisms by the three structural analogs whose formulas are given is overcome in each case by the presence of an excess of biotin, but the three differ among themselves in antibiotin potency and likewise show some differences with different microorganisms. Dethiobiotin for example inhibits the growth of *L. casei* but for yeast is a growth stimulant, i.e., it replaces biotin. It is probable that this is due to the ability of the yeast cell to convert dethiobiotin into biotin. Biotin sulfone is inhibitory to the growth of both yeast and *L. casei*, as is imidazolidone caproic acid, and this inhibition is reversed by biotin. It is interesting to note that all of the antibiotins mentioned combine with avidin.

Another group of synthetic structural analogs of biotin has been described by English, *et al.*[17] The most potent in the group, in terms of the inhibition of growth of *L. casei*, has the following structure:



γ-3,4-Ureylene-cyclohexyl-butyric acid

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[17] English, Clapp, Cole, Halverstadt, Lampen, and Roblin: *J. Am. Chem. Soc.*, 67, 295 (1945).

Other members of the series differ from the structure shown in the type of main ring system and the length of the side chain. Practically all of the compounds described act as antibiotins against either yeast or *L. casei*, but with varying effectiveness, and in every case the inhibition of growth is overcome by the presence of sufficient biotin.

ANALOGS OF HORMONES

Relatively little is known concerning the existence of structural antagonism among the various hormones of known structure. The various protein hormones are of course out of consideration in this connection because their structures are not known; the concept of an "antihormone" for a protein hormone has an immunological connotation rather than one of structural analogy. Among the hormones of known structure, it has been pointed out by Woolley that the naturally occurring estrogens and androgens are structural analogs of each other, and it is suggested that the known instances of biological antagonism between these two groups of compounds may be related to structural similarity and competitive properties. Woolley has also cited as an instance of structural antagonism the interesting findings of Kuhn[18] in connection with the sex hormones of algae. Kuhn showed that the two sex hormones were not different compounds but *cis* and *trans* modifications of the same compound. The factor determining sex was the ratio between these two forms.

It appears likely, therefore, that as further knowledge is gained in the field of hormone chemistry and physiology, the existence of competitive inhibition and modification of biological action by structural differences may become as firmly established for some of the hormones as it now is for vitamins and specific amino acids.

ANALOGS OF OTHER METABOLITES

In addition to the specific examples which have been cited of metabolic antagonism between naturally occurring metabolites and synthetic structural analogs, the concept has been applied to other classes of compounds of biological importance, such as purines, porphyrins, choline and related compounds, amines, etc. A detailed consideration of these appears unprofitable at this time; references to certain of them will be found in the reviews by Welch and by Woolley cited in the Bibliography and in Martin's monograph.

Successful antidotes for poisonous substances must be able to compete with the receptors in the body which are damaged by the poison. Examples are the counteraction of arsenic poisoning by 2,3-dimercaptopropanol (BAL), and the prevention or reversal of cyanide poisoning by vitamin B_{12a}. This substance (hydroxocobalamin) reacts irreversibly in the animal body to form vitamin B_{12} (cyanocobalamin).[19]

ANTIBIOTICS

The terms *antibiosis* and *antibiotic powers* were coined by Paul Vuillemin in 1889 and were used by him in a broad sense, referring to all instances where one species destroys the life of another to preserve or maintain its own. One example of antibiosis cited by Vuillemin fits exactly, however, the current and more restricted concept. He mentioned the observation that products secreted by the blue pus microbe would hold the anthrax bacillus in check. He went on to predict that by an understanding of symbiotic and antibiotic powers man would learn to dominate disease. Present-day definitions characterize antibiotics as organic chemical substances which are produced by microorganisms and have the capacity in dilute solutions to inhibit the growth of other microorganisms and in some situations to destroy them. Opinion is divided whether or not to classify as antibiotics antibacterial substances of animal or vegetable origin.

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[18] Kuhn: *Angew. Chem.*, **53**, 1 (1940).

[19] Mushett, Kelley, Boxer, and Rickards: *Proc. Soc. Exptl. Biol. Med.*, **81**, 234 (1952).

The blue pus microbe, *Pseudomonas aeruginosa*, the first microorganism in which the property of antibiosis was clearly recognized, actually produces several definitely identified[20] compounds with antibacterial activity. The crude mixture had been tried half a century ago against anthrax infections and had been found too toxic to the host to be of any value in treatment. It was not until 1939, when Dubos reported on tyrothricin (see p. 1314), that interest in antibiotics was again accentuated.

Unlike the common disinfectants and antiseptics, which usually act by irreversibly denaturing or precipitating protein, or by inactivating functional groups (sulfhydryl, iron, etc.) within the cell, the action of antibiotics must be much more subtle since they are apparently harmless in the organisms which produce them but inhibitory or toxic in the susceptible organism. Most antibiotics are primarily bacteriostatic in their action; that is, they do not appear to inhibit the metabolic processes of treated microorganisms except where subdivision and growth are concerned. In some instances, however, substrate utilization may be affected. Some antibiotics are bactericidal, irreversibly destroying the metabolic processes of the susceptible cell. In some cases concentration may determine whether the action is bacteriostatic or bactericidal.

Antibiotics differ considerably in their relative effectiveness against various species of microorganisms, and even against different strains of the same species. In some instances susceptibility to an antibiotic may be considerably modified by variations in the conditions of culture.

The differential susceptibility of various species of microorganisms to a given antibiotic permits the construction of a bacterial "spectrum" for the antibiotic, in which the relative effectiveness is determined for a standardized series of test organisms. Such a spectrum may be useful in comparing two supposedly identical or dissimilar antibiotics and in comparing synthetic compounds or derivatives with the naturally occurring antibiotic.

Antibiotics also differ considerably in their relative toxicity to animals and in their effectiveness *in vivo* as compared with *in vitro*. Effectiveness *in vitro* may be modified by the presence in the host of means for metabolizing or destroying the antibiotic.

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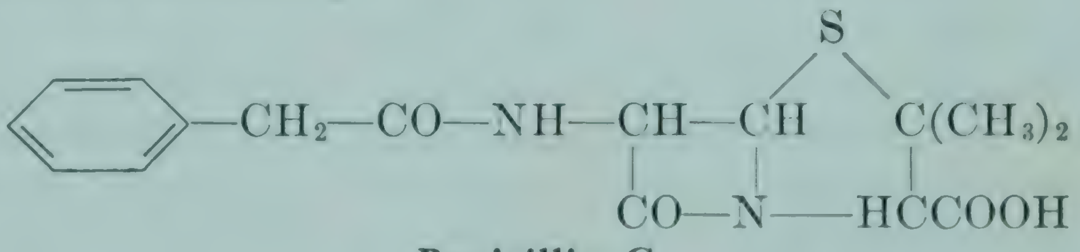
[20] Wells: *J. Biol. Chem.*, **196**, 331 (1952).

The Penicillins. In 1929 Fleming reported[21] that a certain species of mold, later recognized to be a strain of *Penicillium notatum* and first encountered as an accidental contaminant of bacteriological plate cultures, produced a soluble diffusible substance which inhibited the growth of the common *Staphylococcus aureus* and certain (but not all) other microorganisms in culture media. Fleming was able to concentrate this substance from cultures of the mold, and called the material penicillin. It is now known that the original penicillin was a mixture of several similar substances which are called as a class the penicillins.

Fleming showed that his penicillin preparation could be used for the differential separation of resistant and nonresistant species of microorganisms in culture media, and that it was nontoxic to animals. He suggested its possible use in the treatment of infections due to penicillin-sensitive organisms.

These observations however attracted little further attention until about ten years later, when Dubos[22] published his striking results on the antibacterial properties of tyrothricin (see p. 1314). It is probable that these results contributed significantly to a reawakening of interest in the antibacterial possibilities of penicillin. In 1940 Chain, Florey, *et al.*,[23] reported the preparation of penicillin in impure form, with some studies on its toxicity to animals and therapeutic value in experimental infections in mice. This was followed by a more extensive study on methods for large-scale production, partial purification, and assay, and further studies on toxicity and therapeutic value in animals and man. Similar studies on the chemotherapeutic value of penicillin were reported by Dawson, Hobby, Meyer, and Chaffee[24] at about the same time. Developments since then have made penicillin by far the most widely used and important of all the antibiotics thus far known.

Of the various known penicillins, the most important at the present time is the one named penicillin-G (penicillin-II in Britain). The structure of penicillin-G is as follows:



Penicillin-G

As a class, the penicillins all have the composition represented by R—CO—NH—C_7H_9OSN—COOH, where R varies. In penicillin-G for example, R = $C_6H_5CH_2$— (the benzyl group); the relationship among various natural penicillins is indicated in the table on page 1313.

It has been suggested that the various penicillins be designated by names which include the name of the major differentiating group (the R group in the table below). According to this suggestion, penicillin-G becomes benzylpenicillin, penicillin-X is *p*-hydroxybenzylpenicillin, etc.

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[21] Fleming: *Brit. J. Exptl. Path.*, **10**, 226 (1929).

[22] Dubos: *J. Exptl. Med.*, **70**, 1 (1939); Dubos and Cattaneo: *J. Exptl. Med.*, **70**, 249 (1939).

[23] Chain, Florey, Gardner, Jennings, Orr-Ewing, Sanders, and Heatley: *Lancet*, **2**, 226 (1940); Abraham, Chain, Fletcher, Gardner, Heatley, Jennings, and Florey: *Lancet*, **2**, 177 (1941).

[24] Dawson, Hobby, Meyer, and Chaffee: *J. Clin. Invest.*, **20**, 434 (1941).

As a class the penicillins are moderately strong monobasic organic acids, soluble in water and in organic solvents such as alcohol, ether, amyl acetate, etc. The sodium salt, which is quite soluble in water and alcohol, is the usual commercial form. Neutral aqueous solutions of the sodium salt will retain their activity for several days if stored in the cold; acid, alkaline, and alcoholic solutions rapidly become inactive.

THE NATURAL PENICILLINS

Name	*Type Structure:* $R—CO—NH—C_7H_9OSN—COOH$	
	R	*Name of R*
Penicillin-F	$CH_3·CH_2·CH{=}CH·CH_2—$	Δ^2-pentenyl
Dihydropenicillin-F	$CH_3·CH_2·CH_2·CH_2·CH_2—$	*n*-amyl
Penicillin-G	$C_6H_5CH_2—$	benzyl
Penicillin-K	$CH_3·CH_2·CH_2·CH_2·CH_2·CH_2·CH_2—$	*n*-heptyl
Penicillin-X	$HO·C_6H_4·CH_2—$	*p*-hydroxybenzyl

All of the penicillin available commercially is obtained from natural sources, i.e., from cultures of mold, of which special strains of *P. notatum* and *P. chrysogenum* have been most commonly employed. About 30 other variants of penicillin have been produced biosynthetically by the growth of *Penicillium* in the presence of specific organic acid or amide precursors.[25] These penicillins are all antibacterial *in vitro*, and some of them have been used therapeutically, with particular value in patients who have developed sensitivity to benzylpenicillin.

Penicillins inhibit and in higher concentrations kill actively growing cells of susceptible strains. Inhibitory or lethal action on resting or non-multiplying cells is insignificant. By the use of radioactive penicillin, fixation of penicillin by a susceptible organism (*Micrococcus pyogenes* var. *aureus*) was demonstrated to be chiefly in the bacterial cell wall.[26] Penicillin does not enter yeast cells, which are unaffected by its antibiotic activity. Staphylococci can be grown in high concentrations of penicillin, provided these concentrations are low in proportion to bacterial mass. Under these cultural conditions, the conversion of amino acids to bacterial protein was inhibited.[27] Instead, extracellular polypeptides were produced in approximately equivalent amounts. Penicillins also block the uptake of glutamic acid by susceptible staphylococcal strains during active growth. The formation of a precursor of pentose nucleic acid is also delayed. None of these effects has been clearly established as an example of antagonism to a specific metabolite. The structural similarity of benzylpenicillin to glutathione has been noted,[28] but the evidence that penicillin may act as a glutathione antagonist is scant and inconsistent.

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[25] Behrens, Corse, Edwards, Garrison, Jones, Soper, Abeele, and Whitehead: *J. Biol. Chem.*, **175**, 793 (1948); Volini, Shlaes, and Felsenfeld: *J. Am. Med. Assoc.*, **143**, 794 (1950).

[26] Few, Cooper, and Rowley: *Nature*, **169**, 283 (1952).

[27] Hotchkiss: *J. Exp. Med.*, **91**, 351 (1950).

Penicillin may be administered orally or by injection, usually intramuscularly. Questions of dosage, form of administration, and choice of penicillin as compared with other therapeutic agents will not be considered here. The monograph of Welch and Lewis gives the bacterial "spectra" and the details of therapeutic application of penicillin and other antibiotics. Penicillins are nontoxic to animal tissues in any except very large doses; for example, in ordinary concentrations they have no demonstrable effect on the metabolic characteristics of those isolated animal tissues which have been thus far investigated. They differ among themselves, however, in their action on microorganisms; penicillin-G, for example, is most effective clinically, while penicillin-K is much less useful. Lack of recognition of this fact led to some confusion concerning the therapeutic effectiveness of commercial penicillin preparations during the early stages of their clinical application. A wide variety of organisms, mostly Gram-positive but including some Gram-negative, are susceptible to the action of penicillin.

Gramicidin and Tyrocidine. The antibiotic preparation obtained by Dubos from cultures of *B. brevis* and called tyrothricin proved on further study to consist of two separate compounds, called gramicidin and tyrocidine. They may be separated from tyrothricin by treatment with acetone-ether mixture, in which gramicidin is soluble but tyrocidine is insoluble. Gramicidin may be recrystallized from acetone, and tyrocidine hydrochloride from acidified alcohol. Both of these substances are polypeptides of fairly low molecular weight but as yet unknown structure. They are resistant to the action of proteolytic enzymes. On hydrolysis they yield mixtures of amino acids, all of which have presumably been identified (see below). It is interesting to note that certain of these amino-acids are of the D configuration, in contrast with the hydrolytic products of animal proteins, where the L configuration prevails.

Gramicidin is insoluble in water, slightly soluble in ether, and readily soluble in acetone and alcohol. Estimates of its molecular weight vary; on the basis of the amino acid composition a value of approximately 2800 has been proposed. On hydrolysis, gramicidin yields five known amino acids and the basic compound ethanolamine. These components of the gramicidin molecule, and their estimated molecular ratios, are as follows: D-leucine, 6; L-tryptophan, 6; DL-valine, 5; L-alanine, 3; glycine, 2; ethanolamine, 2; making a minimum of 24 residues present. The valine was probably racemized during the hydrolysis; if it were all present in the D form, the leucine and valine would account for the approximately 45 per cent of D-amino acids found present in gramicidin by the use of the enzyme D-deaminase (D-amino acid oxidase).[29] There are no free carboxyl or amino groups, so that a cyclic structure is probable.

Gramicidin is inhibitory to all Gram-positive organisms except acid-fast bacilli, but is entirely inactive against Gram-negative bacilli. It does not destroy the respiration of susceptible cells; in particular instances it may be highly bacteriostatic but not at all bactericidal, thus indicating that it acts rather as a metabolic inhibitor than as a protoplasmic poison. The therapeutic value of gramicidin is limited by its relative toxicity to animals and its low solubility in water, penicillin for example being much superior in both respects. It has, however, found some application in the topical treatment of infected wounds.

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[28] Grevenstuk: *Science*, **114**, 74 (1951).

[29] Lipmann, Dubos, and Hotchkiss: *J. Biol. Chem.*, **141**, 163 (1941).

Tyrocidine is a polypeptide containing free amino groups (probably the δ-amino group of the ornithine present, see below) and forms a crystalline hydrochloride, which is insoluble in water, acetone, and ether but soluble in alcohol, from which it may be crystallized. On hydrolysis it yields the amino acids phenylalanine, leucine, proline, valine, tyrosine, ornithine, glutamic acid, aspartic acid, and tryptophan. Of these, the phenylalanine has the D configuration while all the rest (with the possible exception of tryptophan) have the common L configuration. It is interesting to note that the amino acid valine occurs in one configuration in tyrocidine and in the opposite configuration in gramicidin. The proof of the existence of ornithine in the tyrocidine molecule is probably the first instance of the discovery of this amino acid as a primary constituent of polypeptide chains in nature.

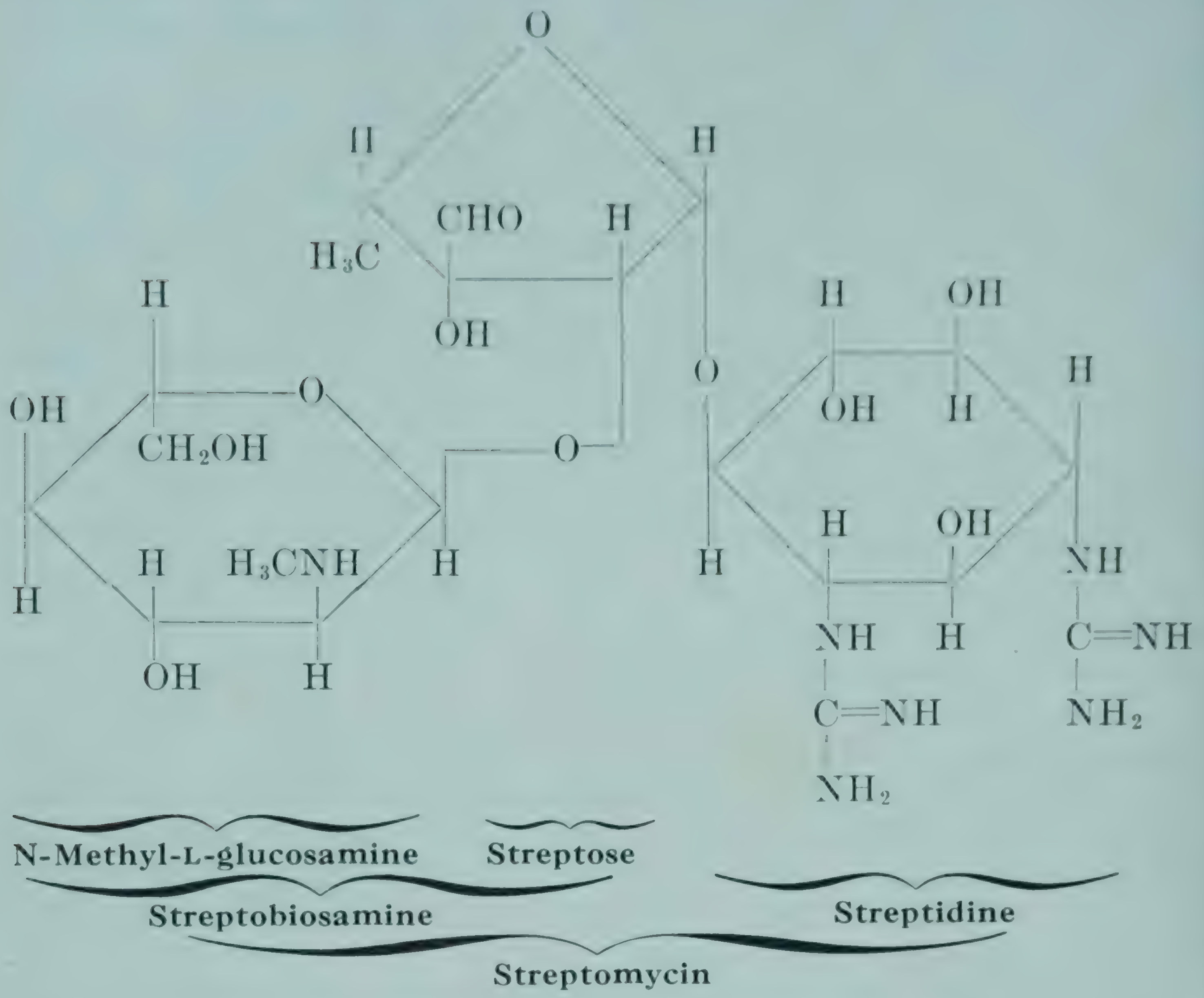
Tyrocidine is strongly bactericidal for various Gram-positive and some Gram-negative organisms *in vitro*. It is inactivated *in vivo* by blood plasma and body fluids in general, and so contributes little to the clinical value of tyrothricin. The importance of tyrothricin in medicine is indicated by its inclusion in the United States Pharmacopeia XIV. Its clinical applications are limited, however, by its toxicity, which prevents systemic use. The chief method of administration is by local application or by instillation into body cavities.

Streptomycin. This antibiotic, obtained from cultures of the actinomycete *Streptomyces griseus*, was first characterized by Schatz, Bugie, and Waksman in 1944. Its discovery was the result of a systematic study of organisms antagonistic to Gram-negative bacteria.

Streptomycin is a water-soluble, crystallizable, organic base, with the structure shown in the formula on page 1316. Its solutions are administered by injection, since absorption from the gastrointestinal tract is minimal.

Although streptomycin was originally introduced as a therapeutic agent against infection with Gram-negative organisms in general, the so-called broad-spectrum antibiotics (which will be considered later) have replaced many of its earlier uses. Against the tubercle bacillus, however, streptomycin remains the most useful of the antibiotics. Both streptomycin and a derivative, *dihydrostreptomycin*, are toxic, affecting the eighth cranial nerve. Streptomycin tends to cause damage to vestibular function and dihydrostreptomycin to auditory function. Use of a mixture of the two has minimized damage.

Concerning the effect of streptomycin on bacterial metabolism, observations with *E. coli* show that the antibiotic inhibits a condensation between oxalacetic and pyruvic acids which precedes their oxidation by a path differing from the usual citric acid cycle. Certain variants of *E. coli* which have been made resistant to (or dependent upon) streptomycin do not utilize these metabolites appreciably, but depend upon other metabolic reactions.[30] Numerous other possible mechanisms of antibacterial action have been noted.



Neomycin, from *Streptomyces fradiae,* is a mixture of water-soluble basic substances, effective against the tubercle bacillus along with a wide variety of Gram-positive and Gram-negative organisms. Its clinical use is limited by a toxic action leading to focal necrosis in the renal tubules.

Bacitracin, from *Bacillus licheniformis* (closely related to *B. subtilis*) is a polypeptide, comparable in a general way with certain other polypeptides of microbial origin—polymyxin, subtilin, and eumycin—which also are antibiotics. Bacitracin has had wide application in the treatment of skin infections, one of its advantages being that it seldom induces allergic reactions. All of the antibiotic polypeptides show some degree of toxicity to the kidney, which limits their systemic use.

Broad-spectrum antibiotics. This group of antibiotics is characterized by activity against a great number of different pathogenic microorganisms, including certain rickettsiae and large viruses. *Chlortetracycline* (Aureomycin) and *oxytetracycline* (Terramycin), from *Streptomyces aureofaciens* and *rimosus,* respectively, are quite similar in structure. Both are relatively nontoxic and are commonly given by mouth, although intravenous use is possible.

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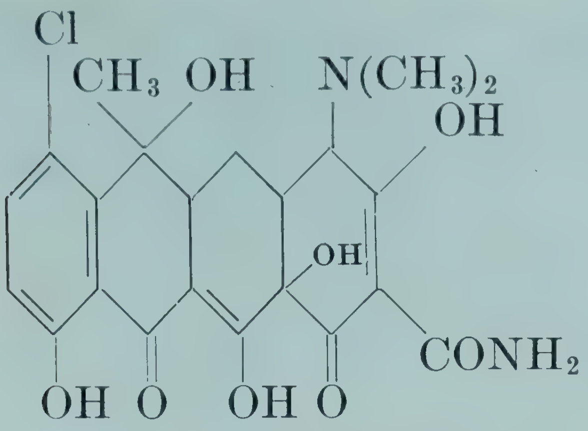
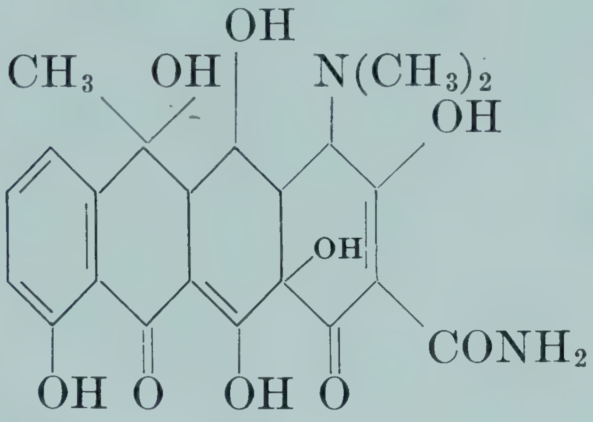
[30] Umbreit, Smith, and Oginsky: *J. Bact.*, **61**, 595 (1951); Umbreit: *Trans. N. Y. Acad. Sci.*, Ser. II, **15**, 12 (1952).

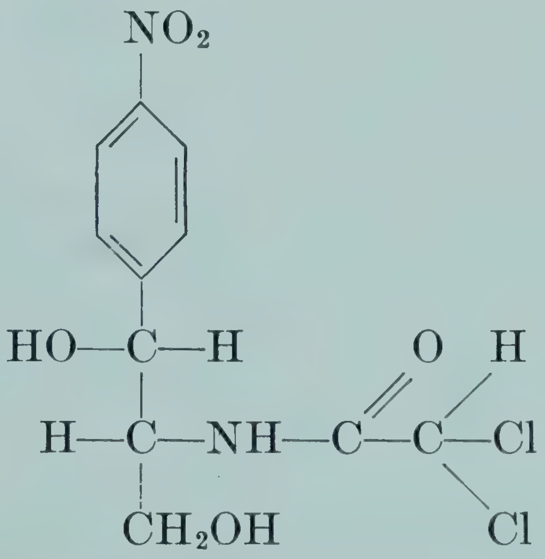


Chlortetracycline
(Aureomycin)



Oxytetracycline
(Terramycin)

Chloramphenicol was originally identified as a naturally occurring antibiotic, elaborated by a species of *Streptomyces*. It is now produced synthetically. Chloramphenicol is the first nitrobenzene compound to be found in living cells.



Chloramphenicol

It is a noncompetitive antagonist of phenylalanine. Woolley[31] has emphasized that noncompetitive antimetabolites are more effective antibiotics, since their activity is not canceled by a simple increase in concentration of the specific metabolite. Chloramphenicol may be given orally or intravenously, and is of particular therapeutic value in typhoid fever and against certain rickettsiae, spirochetes, and viruses. Some degree of calculated risk is involved in its use, since depression of bone marrow function has been reported in a small fraction of cases treated with this agent.

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[31] Woolley: *J. Biol. Chem.*, **185**, 293 (1950).

All three of the broad-spectrum antibiotics have the property in common of inhibiting the synthesis of protein in susceptible bacterial cells. Chlortetracycline will dissociate phosphorylation from oxidation in animal tissue or mitochondrial preparations, and at higher concentration will inhibit both processes. At similar concentrations, terramycin shows these actions only minimally.[32]

Antibiotics in Feeds. The crude residues of *Streptomyces aureofaciens* and of other organisms grown for the production of antibiotics were introduced into the rations of poultry and pigs as a source of vitamin B_{12}. The growth-promoting effect of these antibiotic residues was found to be greater than could be accounted for by their B_{12} content. A similar impetus to growth of pigs, chicks, and poults could be observed on the addition of penicillin, streptomycin, chlortetracycline, or oxytetracycline to a diet adequate by all recognized standards. The hypothesis that these antibiotics are themselves essential growth factors is unlikely on account of the diversity of their chemical structure, and because a similar effect can be observed upon administration of sulfonamides, phenylarsonic acid and other nonbiological antibacterial agents. It is now understood that the growth stimulus depends upon the more or less selective antibacterial action of these substances, which permits the multiplication of certain bacteria which produce B vitamins and possibly other growth factors, meanwhile suppressing other organisms which would compete with these beneficial organisms or even destroy the growth factors produced by them. In some instances actual infections, previously unrecognized, have been shown to be suppressed by the antibiotics included in the rations.

DETERMINATION OF PENICILLIN

1. Method of Vincent and Vincent:[33] ***Principle.*** A disk of filter paper is saturated with the solution to be analyzed, which has been diluted if necessary so as to contain approximately 1 unit per ml. The disk is placed on a nutrient agar culture plate impregnated with the test organisms and incubated at 37° C. If the solution contains penicillin, after incubation a clear zone of inhibition of colony formation surrounds the disk. The diameter of this zone is measured and the penicillin content determined by reference to a calibration curve showing the relation between inhibition zone diameters and known amounts of penicillin.

Procedure. **The test organism (*Staphylococcus aureus* H is recommended) is transferred from an agar slant twice through peptone broth for 24-hour periods at 37° C. The second transfer is held at 5° for 16 to 18 hours.**

Pipet 10 ml. of nutrient agar into uniform flat-bottomed petri dishes and incubate at 37° C. for 16 to 18 hours. Transfer to a refrigerator for at least one hour. When the plates are ready, flood each one with 1 ml. of the refrigerated culture of test organisms. Remove the excess with a capillary pipet. Place the plates at 37° C. for one hour to dry. At this point it is necessary to use wooden racks which support the top half of the petri dish above the bottom half so that there is about ½ inch clearance. When the plates are dry, store in an inverted position in the refrigerator for at least one hour.

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[32] Regna: *Trans. N. Y. Acad. Sci.*, Ser. II. **15**, 12 (1952).

[33] Vincent and Vincent: *Proc. Soc. Exp. Biol. Med.*, **55**, 162 (1944). Similar methods were described almost simultaneously by Epstein, Foley, Perrine and Lee: *J. Lab. Clin. Med.*, **29**, 319 (1944), and by Sherwood, Falco, and de Beer: *Science*, **99**, 247 (1944).

Sterilize the filter-paper disks[34] by dry heat and immerse them for 30 seconds in the solution to be tested. Remove each disk from the fluid with sterile forceps, shake off excess fluid, and place flat on the seeded agar plate. Three disks, evenly spaced, may be placed on a plate, and it is suggested that the plates be run in triplicate for greater accuracy. One of the disks on a plate may serve as a standard penicillin control.

Incubate the prepared plates (not inverted) at 37° C. for 14 hours. The plates should be placed on a wooden block in the incubator to prevent excess condensation. After the incubation period, measure to the nearest mm. the diameter of the zone of inhibition around each disk.[35] Average the results for each unknown.

CALCULATION. The penicillin content of the sample is equal to that of a standard solution of penicillin which gives the same diameter of inhibition zone under comparable conditions. This is best established by reference to a previously prepared calibration curve relating known amounts of penicillin to zone diameters. To prepare such a curve, set up a series of solutions of penicillin in amounts ranging from 4 to 0.1 units per ml., and carry out the procedure as described for the unknown on each of these solutions. Plot a curve with zone diameters in mm. on the y axis against units of penicillin per ml. on the x axis. A curve similar to that shown in Fig. 293 should be obtained. It is advisable to check this curve at intervals with standard penicillin solutions, and to reconstruct it if necessary.

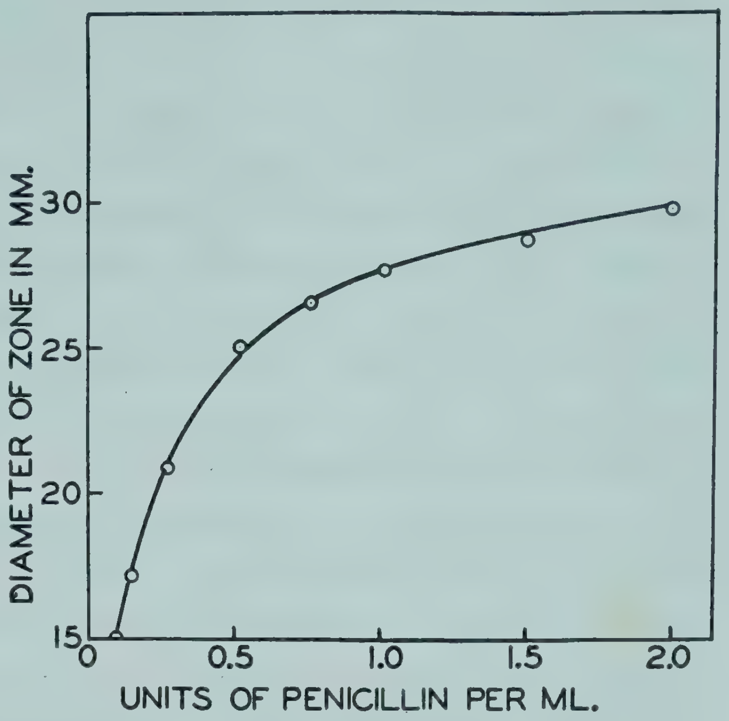

FIG. 293. TYPE OF CALIBRATION CURVE OBTAINED IN PENICILLIN DETERMINATION BY METHOD OF VINCENT AND VINCENT.

Interpretation. This method is a slight modification of the original method of Chain, Florey, *et al.* (*loc. cit.*) developed at Oxford in England and called the "Oxford cup" method, in which the solution to be tested is placed in small glass cylinders partially immersed in the seeded agar; this solution then diffuses out beneath the cup rim into the agar to produce a zone of inhibition similar to that described here. A unit of penicillin (formerly called the Oxford unit and now known as the Florey unit) was defined as that amount which produced a zone of inhibition 24 mm. across under the conditions of the cup assay. Such a unit is clearly subject to variation from laboratory to laboratory and even in the same laboratory unless assay procedures are rigorously controlled. With the availability of pure crystalline sodium penicillin-G, it is possible to define a penicillin unit in terms of this substance. The Federal Food and Drug Administration of the United States has adopted the following relationship: 1 mg. of pure sodium penicillin-G is equivalent to 1667 units of penicillin. Thus 1 unit equals 0.6 μg. of the sodium salt.

The disk method described here has been found to give satisfactory results on blood serum, spinal fluid, urine, and other solutions whose penicillin activity is to be established. For greatest accuracy the solution to be tested should contain between 0.1 and 4 units per ml.

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[34] Schleicher and Schull No. 470, ½ inch in diameter.

[35] A bacteriological "colony counter," equipped with a magnifying glass and a built-in millimeter scale, is very satisfactory for this measurement.

2. Other Methods. Rammelkamp[36] has described a procedure for penicillin assay in which the fluid to be analyzed is incubated at various dilutions with a standard inoculum of hemolytic streptococci in the presence of erythrocytes. Dilutions of a standard penicillin solution are treated in the same way. From the relative dilutions of sample and standard required to inhibit the hemolytic action of the test organisms, the penicillin content is obtained. This method, although not so accurate as the one described above, has given satisfactory results on whole blood, erythrocytes, urine, spinal fluid, exudates, and joint fluid. Hiscox[37] has described a colorimetric procedure, applicable to benzylpenicillin only, for the assay of batches of commercial penicillin. This method involves nitration of the benzene ring, reduction, diazotization, and coupling with a color reagent. A turbidimetric method has been reported[38] which is applicable to the measurement of any of the commonly used antibiotics in blood or body fluids.

For techniques of streptomycin assay, and of tests of organisms for sensitivity to antibiotics, consult the *Diagnostic Procedures and Reagents* of the American Public Health Association. Sensitivity tablets and disks impregnated with varying concentrations of different antibiotics are available from biological supply houses, and each manufacturer recommends procedures and media for use with his product.

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[36] Rammelkamp: *Proc. Soc. Exp. Biol. Med.*, **51**, 95 (1942).

[37] Hiscox: *Analytical Chem.*, **22**, 722 (1950).

[38] Whitlock, Hunt, and Tashman: *J. Lab. Clin. Med.*, **37**, 155 (1951).

Appendix

I. REAGENTS AND SOLUTIONS

REAGENTS FOR PARTICULAR METHODS ARE DESCRIBED IN THE TEXT AND FOOTNOTES FOR THESE METHODS. SEE INDEX

Acid Digestion Mixture. To 50 ml. 5 per cent copper sulfate solution add 300 ml. 85 per cent phosphoric acid and mix. Add 100 ml. concentrated sulfuric acid (NH_3-free) and mix. Keep in a glass-stoppered bottle, protected against the absorption of ammonia from laboratory atmosphere.

Alcohol-Ether Mixture. Mix three volumes 95 per cent redistilled alcohol and 1 volume redistilled ether.

Alizarin. A 1 per cent solution of alizarin monosodium sulfonate in water.

Alkaline Pyrogallate Reagent. Prepare a solution of potassium hydroxide by dissolving 160 g. in 130 ml. water. In 200 ml. of this solution dissolve 10.0 g. pyrogallic acid.

Almén's Reagent. Prepare by dissolving 5 g. tannic acid in 240 ml. 50 per cent alcohol and adding 10 ml. 25 per cent acetic acid.

Aluminum Hydroxide Cream. To a 1 per cent solution of ammonium alum at room temperature add a slight excess of a 1 per cent solution of ammonium hydroxide. Wash by decantation until the wash water shows only the faintest trace of residue on evaporation. For the preparation of other forms of alumina see p. 329.

***o*-Aminobenzaldehyde Reagent.** Mix 3 g. crystalline *o*-nitrobenzaldehyde with 50 g. crystalline ferrous sulfate. Add 75 ml. concentrated ammonia. Heat on a steam bath for one hour. Distil off the *o*-aminobenzaldehyde with steam. The mixture before distillation will keep for two weeks.

Ammoniacal Silver Solution. Dissolve 26 g. silver nitrate in about 500 ml. water, add enough ammonium hydroxide to redissolve the precipitate which forms upon the first addition of the ammonium hydroxide, and make the volume of the mixture up to 1 liter with water.

Ammonium Molybdate Solution. See Molybdate Solution, p. 1328.

Ammonium Thiocyanate Solution. This solution is made of such a strength that 1 ml. of it is equal to 1 ml. of the standard silver nitrate solution mentioned below. To prepare the solution dissolve 12.9 g. ammonium thiocyanate, NH_4SCN, in a little less than a liter of water. In a small flask place 20 ml. of the standard silver nitrate solution, 5 ml. of a cold saturated solution of ferric alum, and 4 ml. nitric acid (sp. gr. 1.2), add water to make the total volume 100 ml., and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a buret until a permanent *red-brown* tinge is produced. This is the end reaction and indicates that the last trace of silver nitrate has been precipitated. Take the buret reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 10 ml. of this solution may be exactly equal to 10 ml. of the silver nitrate solution. Make the dilution and titrate again to be certain that the solution is of the proper strength.

Antifoaming Oil Mixture (Use like caprylic alcohol). To 1 volume crude fuel oil add about 10 volumes toluene.

Asbestos for Suction Filters. The asbestos is shredded, placed in a wide-mouth flask, and covered with 10 per cent HCl. Heat on a water bath for five hours. Filter on a Buchner funnel, wash free from acid, return to the flask, cover with 5 per cent NaOH, and heat on a water bath for three hours. Filter, wash free from alkali, then with dilute acid and finally with water until free from acid. Suspend in a large volume of water, allowing it to settle for five minutes. Pour off the upper two-thirds and discard. Repeat the washing of the desired coarse portion several times until the supernatant liquid remains nearly clear.

Barfoed's Solution. Dissolve 13.3 g. neutral, crystallized copper acetate in 200 ml. water, filter if necessary, and add 1.8 ml. glacial acetic acid.

Barfoed's Solution (Tauber-Kleiner Modification). Dissolve 24 g. copper acetate (Merck, normal, c.p.) in 450 ml. boiling water. If a precipitate forms, do not filter. Immediately add 25 ml. 8.5 per cent lactic acid (Mallinckrodt, U.S.P., 85 per cent) to the hot solution. Shake; nearly all the precipitate will dissolve. Cool, dilute to 500 ml., and after sedimentation filter off the impurities.

Baryta Mixture. A mixture consisting of 1 volume of a saturated solution of barium nitrate and 2 volumes of a saturated solution of barium hydroxide.

Basic Lead Acetate Solution. This solution possesses the following formula:

Lead acetate	180 g.
Lead oxide (litharge)	110 g.
Distilled water to make	1000 g.

Dissolve the lead acetate in about 700 ml. distilled water, with boiling. Add this hot solution to the finely powdered lead oxide and boil for one-half hour with occasional stirring. Cool, filter, and add sufficient distilled water to the filtrate to make the weight 1 kg.

Benedict's Solution. Benedict's modification of the Fehling solution is stable even upon long standing. It has the following composition:

Copper sulfate	17.3 g.
Sodium citrate	173.0 g.
Sodium carbonate	100.0 g.
Distilled water to make	1 liter

With the aid of heat dissolve the sodium citrate and carbonate in about 800 ml. water. Pour (through a folded filter paper if necessary) into a glass graduate and make up to 850 ml. Dissolve the copper sulfate in about 100 ml. water. Pour the carbonate-citrate solution into a large beaker or casserole, and add the copper sulfate solution slowly, with constant stirring, and make up to 1 liter. The mixed solution is ready for use and does not deteriorate upon long standing.

Benedict's Quantitative Sugar Reagent.

Copper sulfate (crystallized)	18.0 g.
Sodium carbonate (crystallized, one-half the weight of the anhydrous salt may be used)	200.0 g.
Sodium or potassium citrate	200.0 g.
Potassium thiocyanate	125.0 g.
Potassium ferrocyanide (5 per cent solution)	5.0 ml.
Distilled water to make a total volume of	1000.0 ml.

With the aid of heat dissolve the carbonate, citrate, and thiocyanate in enough water to make about 800 ml. of the mixture and filter if necessary. Dissolve the copper sulfate separately in about 100 ml. water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool, and dilute to exactly 1 liter. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five ml. of the reagent are reduced by 50 mg. of glucose.

Benedict's Sulfur Reagent.

Crystallized copper nitrate, sulfur-free or of known sulfur content	200 g.
Sodium or potassium chlorate	50 g.
Distilled water to	1000 ml.

Benzidine Solution. Place 4.33 ml. glacial acetic acid in a small Erlenmeyer flask, warm to 50° C., and add 0.5 g. of benzidine. Heat the flask for eight to ten minutes in water at 50°. To the resultant solution add 19 ml. of distilled water. This solution may be kept for several days without deterioration.

Bial's Reagent.

Orcinol	1.5 g.
Concentrated HCl	500 ml.
Ferric chloride (10 per cent)	20–30 drops

α, α'-**Bipyridine Solution.** Dissolve 0.2 g. α, α'-bipyridine (Edwal Laboratories, 732 Federal St., Chicago) in 100 ml. 10 per cent acetic acid.

Biuret Paper (Kantor and Gies). Immerse filter paper in Gies' Biuret Reagent (below), then dry and cut into strips.

Biuret Reagent (Gies). This reagent consists of 10 per cent KOH solution to which 25 ml. 3 per cent $CuSO_4$ solution per liter has been added. This imparts a slight though distinct color to the clear liquid.

Biuret Reagent (Welker). Add 1 per cent copper sulfate solution, drop by drop, with constant stirring, to some 40 per cent sodium hydroxide solution until the mixture assumes a deep blue color.

Black's Reagent. Make by dissolving 5 g. ferric chloride and 0.4 g. ferrous chloride in 100 ml. water.

Boneblack, Purification of. Treat 250 g. commercial boneblack with 1500 ml. 1:4 HCl solution. Boil for half an hour. Filter the boneblack from the acid solution by means of a Buchner funnel and aspirating pump. Wash with hot water until the washings are neutral to litmus paper. Dry and powder.

Bromine Water. Add a few drops of liquid bromine (*Caution: Very corrosive!*) to about 100 ml. H_2O, and shake. Prepare fresh when reagent becomes colorless.

Buffer Solutions, Standard. See pp. 35 to 37.

Carmine-fibrin. Run fibrin through a meat chopper, wash carefully, and place in a 0.5 per cent ammoniacal carmine solution (1 g. carmine dissolved in 1 ml. ammonia and diluted to 200 ml.) for 24 hours or until the maximum coloration of the fibrin (a dark red) is obtained. The fibrin is then washed in water and water acidified with acetic acid. It is preserved under glycerol.

Cleaning Solution. To a few grams of sodium bichromate crystals dissolved in a minimum quantity of water, add about 500 ml. concentrated sulfuric acid (technical grade is satisfactory).

Cochineal Solution. A saturated solution of cochineal in 30 per cent alcohol.

Congo Red. Dissolve 0.5 g. Congo red in 90 ml. water and add 10 ml. 95 per cent alcohol.

Congo Red-Fibrin. This may be prepared by placing fibrin in faintly alkaline Congo red solution and heating to 80° C. The fibrin is then washed and preserved under glycerol.

Copper Sulfate Solutions for Specific Gravity Determination.

A. SATURATED COPPER SULFATE SOLUTION. Place 4 lb. "fine crystals," or pulverized $CuSO_4 \cdot 5H_2O$, in a 4-liter bottle, and add about 2.5 liters distilled water. Stopper and shake vigorously for five minutes. Immediately at the close of the shaking period, insert a thermometer into the solution and record the temperature to the nearest half degree Centigrade. When this has been done, immediately decant the supernatant solution from the excess solid, then clarify by filtration through cotton or a dry filter paper into a clean dry bottle. This solution, which has been saturated with copper sulfate at a known temperature and is therefore of known composition, is used at once to make up a stock copper sulfate solution of sp. gr. 1.100.

B. STOCK COPPER SULFATE SOLUTION OF SP. GR. 1.100. From the table (p. 1325) determine the volume of saturated copper sulfate solution which is to be diluted to 1 liter to prepare a solution of sp. gr. 1.100. Measure out the indicated volume of saturated copper sulfate solution into a 500-ml. graduated cylinder. Pour the cylinder contents into a 1-liter volumetric flask, allowing the cylinder to drain for 30 seconds. Dilute the flask contents to the mark with distilled water, stopper, and mix well by inversion. Because of a contraction in volume, the meniscus of the solution will now be somewhat below the mark. Allow to stand for one minute for drainage, then add enough distilled water to bring the meniscus again to the mark, stopper, and again mix well by inversion. Pour into a clean dry 4-liter bottle. Rinse the flask with distilled water, discarding the rinsings, and repeat the above preparation three more times, thus preparing 4 liters stock copper sulfate solution of sp. gr. 1.100.

This stock solution, used for the preparation of the standards described below, should be labeled with its temperature at the time of preparation, since it may be used indefinitely provided the temperature at the time of use is within 5° C. of the temperature at the time of preparation.

VOLUME OF SATURATED COPPER SULFATE SOLUTION TO DILUTE TO 1 LITER TO PREPARE STOCK SOLUTION OF $D^{25}_{25} = 1.1000$

Temperature of the Saturated Solution at the Time of Saturation		*Volume of Solution to Dilute to 1 Liter*	*Temperature of the Saturated Solution at the Time of Saturation*		*Volume of Solution to Dilute to 1 Liter*	*Temperature of the Saturated Solution at the Time of Saturation*		*Volume of Solution to Dilute to 1 Liter*
° C.	*° F.*	*Ml.*	*° C.*	*° F.*	*Ml.*	*° C.*	*° F.*	*Ml.*
10.0	50.0	587	20.0	68.0	489	30.0	86.0	424
10.5	50.9	581	20.5	68.9	485	30.5	86.9	421
11.0	51.8	575	21.0	69.8	481	31.0	87.8	418
11.5	52.7	569	21.5	70.7	477	31.5	88.7	415
12.0	53.6	563	22.0	71.6	474	32.0	89.6	412
12.5	54.5	557	22.5	72.5	470	32.5	90.5	410
13.0	55.4	552	23.0	73.4	466	33.0	91.4	407
13.5	56.3	546	23.5	74.3	463	33.5	92.3	404
14.0	57.2	541	24.0	75.2	459	34.0	93.2	401
14.5	58.1	536	24.5	76.1	456	34.5	94.1	398
15.0	59.0	531	25.0	77.0	453	35.0	95.0	395
15.5	59.9	527	25.5	77.9	450	35.5	95.9	392
16.0	60.8	522	26.0	78.8	446	36.0	96.8	389
16.5	61.7	518	26.5	79.7	443	36.5	97.7	387
17.0	62.6	514	27.0	80.6	440	37.0	98.6	384
17.5	63.5	509	27.5	81.5	438	37.5	99.5	381
18.0	64.4	505	28.0	82.4	435	38.0	100.4	378
18.5	65.3	501	28.5	83.3	432	38.5	101.3	374
19.0	66.2	497	29.0	84.2	429	39.0	102.2	371
19.5	67.1	493	29.5	85.1	427	39.5	103.1	368
20.0	68.0	489	30.0	86.0	424	40.0	104.0	365

c. STANDARD COPPER SULFATE SOLUTIONS OF KNOWN SPECIFIC GRAVITY. A 100-ml. portion of copper sulfate solution of any desired sp. gr. between 1.008 and 1.075, accurate to ±0.0003, may be prepared from the stock solution of sp. gr. 1.100 as follows: measure from a buret into a 100-ml. volumetric flask a volume of stock solution *less by 1 ml.* than the value represented by the second and third decimal places of the desired sp. gr. (For instance, if the desired sp. gr. is 1.055, 54 ml. of stock solution are taken; for a sp. gr. of 1.017, 16 ml. are taken and so on.) After measuring out the stock solution, dilute the contents of the 100-ml. flask to the mark with distilled water and mix well. This solution may be used at any temperature within 15° to 20° C. of the temperature at preparation. Smaller portions than 100 ml. may be prepared by using proportionately reduced volumes.

Cross and Bevan's Reagent. Prepare by combining 2 parts of concentrated hydrochloric acid and 1 part of zinc chloride, by weight.

Digitonin Solution. Dissolve 1 g. digitonin (Hoffmann-La Roche, Inc., Nutley, N. J.) in 1 liter of distilled water and place in a refrigerator for 24 hours. Filter. Concentrate the entire filtrate and make up to exactly 500 ml. (To hasten evaporation draw a rapid stream of air, filtered through cotton, over the surface of the solution in a tared flask, which is immersed in a boiling water bath until the desired weight is reached.)

Duponol C. (E. I. du Pont de Nemours and Co., Wilmington, Delaware). Sodium salt of a sulfuric ester of lauryl alcohol.

Ehrlich's Aldehyde Reagent. A solution of *p*-dimethylaminobenzaldehyde acidified with HCl.

Ehrlich's Diazo Reagent. Two separate solutions should be prepared and mixed in definite proportions when needed for use.

(*a*) 5 g. sodium nitrite dissolved in 1 liter distilled water.

(*b*) 5 g. sulfanilic acid and 50 ml. hydrochloric acid in 1 liter distilled water.

Solutions (*a*) and (*b*) should be preserved in well-stoppered vessels and mixed in the proportion 1:50 when required. It is said that greater delicacy is secured by mixing the solutions in the proportion 1:100. A satisfactory modification of this reagent, containing less acid, is described on p. 593. The sodium nitrite deteriorates upon standing and becomes unfit for use in the course of a few weeks.

Esbach's Reagent. Dissolve 10 g. picric acid and 20 g. citric acid in 1 liter water.

Exton's Reagent. Dissolve 200 g. $Na_2SO_4 \cdot 10H_2O$ in 800 ml. water. Cool to 35° C. and add 50 g. sulfosalicylic acid. Dissolve and dilute to 1 liter.

Fehling's Solution. Fehling's solution is a mixture of copper sulfate solution and alkaline tartrate solution, prepared as follows:

Copper sulfate solution = 34.65 g. copper sulfate dissolved in water and made up to 500 ml. *Alkaline tartrate solution* = 125 g. potassium hydroxide and 173 g. Rochelle salt dissolved in water and made up to 500 ml.

These solutions should be preserved separately in rubber-stoppered bottles and mixed in equal volumes when needed for use. This is done to prevent deterioration.

Ferric Hydroxide (Oxide) Suspension. Dissolve 100 g. ferric ammonium sulfate in 200 ml. water. Pour all at once with vigorous stirring into 800 ml. of a mixture containing 10.8 g. ammonia and 27.5 g. ammonium sulfate. Stir vigorously for one-half hour. Make to 4 liters with water. Let settle and decant. Repeat washing about 12 times, adding a few ml. of concentrated ammonia to first two wash waters.

Folin-McEllroy Reagent. Dissolve 100 g. sodium pyrophosphate, 30 g. disodium phosphate and 50 g. dry sodium carbonate in approximately 1 liter of water by the aid of a little heat. Dissolve separately 13 g. copper sulfate in about 200 ml. water. Pour the copper sulfate solution into the phosphate-carbonate solution and shake.

Furfural Solution. Add 1 ml. furfural to 1000 ml. distilled water.

Fusion Mixture. 20 g. sodium carbonate plus 10 g. potassium nitrate.

Guaiac Solution. Dissolve 0.5 g. guaiac resin in 30 ml. 95 per cent alcohol.

Gum Ghatti Solution. Fill a liter cylinder with cold water. Just below the surface suspend a wire screen on which are placed 20 g. soluble gum ghatti. Let stand 18 to 24 hours. Remove the screen and filter, or strain through clean cloth.

Günzberg's Reagent. Dissolve 2 g. phloroglucinol and 1 g. vanillin in 100 ml. 95 per cent alcohol.

Haines' Solution. This solution may be prepared by dissolving 8.314 g. copper sulfate in 400 ml. water, adding 40 ml. glycerol and 500 ml. 5 per cent potassium hydroxide solution.

Hopkins-Cole Reagent. To 1 liter of a saturated solution of oxalic acid add 60 g. sodium amalgam and allow the mixture to stand until the evolution of gas ceases. Filter and dilute with 2 to 3 volumes of water.

Hopkins-Cole Reagent (Benedict's Modification). Place 10 g. powdered magnesium in a large Erlenmeyer flask and shake up with enough distilled water to liberally cover the magnesium. Now slowly add 250 ml. of a cold, saturated solution of oxalic acid. The reaction proceeds very rapidly and with the liberation of much heat, so that the flask should be cooled under running water during the addition of the acid. The contents of the flask are shaken after the addition of the last portion of the acid and then poured upon a filter, to remove the insoluble magnesium oxalate. A little wash water is poured through the filter, the filtrate acidified with acetic acid to prevent the partial precipitation of the magnesium on long standing, and made up to a liter with distilled water. This solution contains only the magnesium salt of glyoxylic acid.

Hübl's Iodine Solution. A solution of iodine and mercuric chloride used to determine iodine numbers of unsaturated compounds. (See Iodine Solution, Wijs.)

Hydrochloric Acid Standard (0.1 N) Solution. See p. 868.

Hypobromite Solution. The ingredients of this solution should be prepared in the form of *two* separate solutions which may be united as needed.

(*a*) Dissolve 125 g. sodium bromide in water, add 125 g. bromine, and make the total volume of the solution 1 liter.

(*b*) A solution of sodium hydroxide having a specific gravity of 1.25. This is approximately a 22.5 per cent solution.

Preserve both solutions in rubber-stoppered bottles and when needed for use mix 1 volume of solution (*a*), 1 volume of solution (*b*), and 3 volumes of water.

Iodine Solution. Prepare a 2 per cent solution of potassium iodide and add sufficient iodine to color it a deep yellow.

Iodine Solution (0.1 N). Weigh 12.685 g. pure resublimed iodine into a small weighing bottle using a porcelain spatula. Dissolve 18 g. pure KI in about 150 ml. water. Transfer the iodine to a liter flask, washing out the last traces with some of the KI solution, which is then poured into the flask. Stopper and shake occasionally until dissolved. If necessary, a few more crystals of KI may be added to aid solution. Dilute to the mark and mix well. Keep in a glass-stoppered bottle in a cool dark place. Standardize at once against 0.1 N sodium thiosulfate solution. Measure out accurately 25 ml. of the iodine solution into an Erlenmeyer flask, run in sodium thiosulfate until the color is pale yellow, then add a few ml. of a 1 per cent solution of starch (preferably soluble starch) and titrate to disappearance of blue color. Care should be taken near the end point.

Iodine Solution (Wijs). Weigh into a 300-ml. flask 9.4 g. iodine trichloride. Add about 200 ml. glacial acetic acid. Stopper with a cork carrying a $CaCl_2$ tube and heat on the water bath until solution is complete. Rub 7.2 g. iodine to a fine powder in a mortar, wash with glacial acetic acid into a second flask, and heat this in the same way to dissolve the iodine. Pour the contents of both flasks into a liter volumetric flask. Add glacial acetic acid. Measure 10 ml. of the solution into a 500-ml. Erlenmeyer flask. Add 10 ml. 10 per cent KI and about 200 ml. water. Titrate with standard sodium thiosulfate solution and determine the iodine equivalent of 1 ml. of the solution.

Iodine-Zinc Chloride Reagent. Dissolve 20 g. $ZnCl_2$ in 8.5 ml. water and, when cool, introduce the iodine solution (3 g. KI and 1.5 g. I in 60 ml. water) drop by drop until iodine begins to precipitate.

Kraut's Reagent. Dissolve 272 g. KI in water and add 80 g. bismuth subnitrate dissolved in 200 g. HNO_3 (sp. gr. 1.18). Permit the KNO_3 to crystallize out; then filter it off and make the filtrate up to 1 liter with water.

Lead Acetate, Basic. (See Basic Lead Acetate.)

Lime Water. Shake up an excess of calcium oxide or hydroxide with distilled water and leave well-stoppered over night. Decant clear supernatant solution and keep free from CO_2 of air.

Litmus-Milk Powder. Add 1 part powdered litmus to 50 parts dried milk powder. To make a litmus milk *solution*, add 1 part of this powder to 9 parts water.

Lohmann's Reagent. Add 25 ml. concentrated nitric acid to 100 g. mercuric nitrate octahydrate, followed by 25 ml. water. Warm to dissolve.

Lugol's Solution. Dissolve 5 g. iodine and 10 g. potassium iodide in 100 ml. distilled water.

Magnesia Mixture. Dissolve 175 g. magnesium sulfate and 350 g. ammonium chloride in 1400 ml. distilled water. Add 700 g. concentrated ammonium hydroxide, mix thoroughly, and preserve the mixture in a glass-stoppered bottle.

Magnesium Nitrate Solution for Ignition. Dissolve 320 g. calcined magnesia in nitric acid, avoiding an excess of the latter; then add a little calcined magnesia in excess, boil, filter from the excess of magnesia, ferric oxide, etc., and dilute with water to 2 liters.

Methyl Orange. Dissolve 0.1 g. methyl orange in 100 ml. distilled water.

Methyl Red. Saturated solution in 50 per cent alcohol.

Milk of Lime. A 1.5 per cent suspension of CaO in water. To be labeled: "Shake before using."

Millon's Reagent. Digest 1 part (by weight) mercury with 2 parts (by weight) nitric acid (sp. gr. 1.42) and dilute the resulting solution with 2 volumes water.

Molisch's Reagent. A 5 per cent alcoholic solution of α-naphthol.

Molybdate Solution. Dissolve 100 g. molybdic acid in 144 ml. ammonium hydroxide (sp. gr. 0.90) and 271 ml. water; slowly and with constant stirring pour the solution thus obtained into 489 ml. nitric acid (sp. gr. 1.42) and 1148 ml. water. Keep the mixture in a warm place for several days, or until a portion heated to 40° C. deposits no yellow precipitate of ammonium phosphomolybdate. Decant the solution from any sediment and preserve in glass-stoppered bottles.

Mörner's Reagent. Prepare by thoroughly mixing 1 volume formalin, 45 volumes distilled water, and 55 volumes concentrated sulfuric acid.

α-Naphthol Solution. Dissolve 1 g. α-naphthol in 100 ml. 95 per cent alcohol.

Nessler's Reagent: (A) Formula of Folin and Wu. Nessler's solution is an alkaline solution of the double iodide of mercury and potassium ($HgI_2 \cdot 2KI$). Into a 500-ml. Florence flask introduce 150 g. potassium iodide and 100 g. iodine; add 100 ml. water and an excess of metallic mercury, 140 to 150 g. Shake the flask continuously and vigorously for 7 to 15 minutes or until the dissolved iodine has nearly all disappeared. The solution becomes quite hot. When the red iodine solution begins to become visibly pale, though still red, cool in running water and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. The whole operation does not usually take more than 15 minutes. Decant the solution, washing mercury and flask with liberal quantities of distilled water. Dilute the solution and washings to 2 liters. If the cooling was begun in time, the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water and the finished solution can be used at once for nesslerization.

From this stock solution of potassium mercuric iodide prepare final Nessler's solution as follows: Into a flask of at least 5 liters capacity introduce 3500 ml. 10 per cent sodium hydroxide solution, add 750 ml. of the double iodide solution and 750 ml. distilled water, making 5 liters of solution. The 10 per cent NaOH should be made from a saturated solution (containing about 75 g. per 100 ml.) which has been allowed to stand until the carbonate has settled, the clear solution being decanted and used. This solution should have been standardized with an accuracy of at least 5 per cent. Nessler's reagent should be used in the ratio of 10 ml. per 100 ml. of solution to be nesslerized, except where excessive amounts of acids are present, as in direct nesslerization procedures.

The alkalinity of the Nessler's reagent is important and may be checked by titrating with it 20 ml. portions of N HCl. A good end point with phenolphthalein should be obtained at 11 to 11.5 ml. If as little as 9.5 ml. is required, the solution is too alkaline. One ml. of the dilute (1:1) acid digestion mixture should also require 9 to 9.3 ml. Nessler's solution to neutralize it.

(B) Formula of Koch and McMeekin. Dissolve 22.5 g. iodine in 20 ml. water containing 30 g. potassium iodide. After the solution is complete, add 30 g. pure metallic mercury, and shake the mixture well, keeping it from becoming hot by immersing it in tap water from time to time. Continue this until the supernatant liquid has lost all of the yellow color due to iodine. Decant the supernatant aqueous solution and test a portion by adding a few drops thereof to 1 ml. of a 1 per cent soluble starch solution. Unless the starch test for iodine is obtained, the solution may contain mercurous compounds. To the remaining solution add a few drops of an iodine solution of the same concentration as employed above, until a faint excess of free iodine can be detected by adding a few drops thereof to 1 ml. of the starch solution. Dilute to 200 ml. and mix well. To 975 ml. of an accurately prepared 10 per cent sodium hydroxide solution now add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to clear by standing.

(C) Formula of Bock and Benedict. Place 100 g. mercuric iodide and 70 g. potassium iodide in a liter volumetric flask and add about 400 ml. water. Rotate until solution is complete. Now dissolve 100 g.

NaOH in about 500 ml. water, cool thoroughly and add with constant shaking to the mixture in the flask; then make up with water to the liter mark. This usually becomes perfectly clear. When the small amount of brownish-red precipitate which forms settles out, the supernatant fluid is ready to be poured off and used.

Neutral Olive Oil. Shake ordinary olive oil with a 10 per cent solution of sodium carbonate, extract the mixture with ether, and remove the ether by evaporation. The residue is *neutral* olive oil.

Neutral Red. A 1 per cent solution in 50 per cent alcohol.

***p*-Nitrophenol.** A 1 per cent solution in 50 per cent alcohol.

Nylander's Reagent. Digest 2 g. bismuth subnitrate and 4 g. Rochelle salt in 100 ml. of a 10 per cent solution of potassium hydroxide. The reagent should then be cooled and filtered.

Obermayer's Reagent. Add 2 to 4 g. of ferric chloride to a liter of hydrochloric acid (sp. gr. 1.19).

Oxalic Acid Standard (0.1 N) Solution. See p. 867.

Permutit. A synthetic aluminum silicate obtained from the Permutit Company, New York. Only such preparations as pass through a 60-mesh sieve but not through an 80-mesh sieve should be used. It should give off very little dust or turbid material to water and settle in a few seconds. It may be used more than once by washing first with water, then with 2 per cent acetic acid and finally with water again.

Phenol Reagent (Folin and Ciocalteu). See p. 939.

Phenolphthalein. Dissolve 1 g. phenolphthalein in 100 ml. 95 per cent alcohol.

Phenylhydrazine Acetate Solution. This solution is prepared by mixing 1 volume glacial acetic acid, 1 volume water, and 2 volumes phenylhydrazine (the base).

Phenylhydrazine Mixture. This mixture is prepared by combining 2 parts phenylhydrazine hydrochloride and 3 parts sodium acetate *by weight.* These are thoroughly mixed in a mortar. A mixture of better keeping quality may be made by mixing equal weights of the hydrochloride and *anhydrous* sodium acetate.

Phosphomolybdic Acid. Saturate some sodium carbonate solution with pure molybdic acid. Add 1 part crystalline disodium phosphate to 5 parts acid and evaporate to dryness. Fuse in a porcelain dish at a dull red heat. Dissolve the sodium phosphomolybdate in 10 parts water and add nitric acid until the solution turns a golden yellow color.

Phosphotungstic Acid. Dissolve 100 g. sodium tungstate and 60 to 80 g. disodium phosphate in 500 ml. water. Add nitric acid to an acid reaction.

Picric Acid, Pure. Picric acid as purchased contains 10 per cent of added water. By exposure to the air between large filter papers (best in a warm place) the water disappears by evaporation. Dry picric acid may be prepared in this way. If the alkaline picrate formed from this acid gives too deep a color, it may be purified as follows:

A. RECRYSTALLIZATION OF PICRIC ACID FROM ACETIC ACID. Dissolve 100 g. picric acid (previously thoroughly dried at 80 to 90° C.) with the aid of heat in 150 ml. glacial acetic acid in an Erlenmeyer flask and continue the heating on an electric plate until the mixture boils. Pour the hot solution upon a fluted filter contained in a dry funnel which has been previously heated, and collect the filtrate in a dry beaker. Cover with a watch glass and let stand over night at room temperature. If crystallization fails to occur, seed with a small crystal of pure picric acid. At the end of two hours, filter with suction on a hardened filter, and wash with about 35 ml. cold glacial acetic acid. Suck as free from acetic acid as possible and dry at about 80° to 90° C., with occasional stirring, until there is no odor of acetic acid. Conduct operations in a good current of air.

B. SODIUM PICRATE METHOD OF FOLIN. Transfer 500 g. moist picric acid to a Florence flask of 1500 ml. capacity. Add 500 ml. acetone. Shake, with a little warming under hot tap water, until all the crystals have dissolved. Add 20 g. active charcoal ("Norit"). Shake, and filter into another flask. During this filtration keep the funnel closed with a watch glass to prevent evaporation.

Dissolve 250 g. anhydrous sodium carbonate and 100 g. sodium chloride in 2500 ml. warm water in a 4-liter beaker. While stirring with an agateware spoon, add the acetone solution gradually to the alkaline salt solution. When the reaction (CO_2 evolution) is finished, let stand, preferably in cold water, for about 30 minutes, and filter on a large Buchner funnel (diam. 20 cm.). Wash with about 2 liters sodium chloride solution (7 per cent) and suck as dry as possible.

If the original picric acid is of good quality, the sodium picrate on the Buchner funnel will be pure, but it is a little safer to recrystallize it once as follows:

Return the precipitate to the 4-liter beaker and add 2 liters boiling water and 20 g. sodium carbonate. To the resulting hot solution add gradually, with stirring, 150 g. sodium chloride, cool, filter, and wash as before with 7 per cent sodium chloride solution. Then wash once or twice with a more dilute sodium chloride solution (2 per cent) and finally wash once with methyl alcohol to remove most of the remaining chloride and water. Dry, either at room temperature or over a radiator.

TEST FOR THE PURITY OF SODIUM PICRATE. Make 100 ml. of a 3 per cent solution. Transfer 5 ml. and 10 ml. to test tubes graduated at 25 ml. Dilute each to about 22 ml., add 2 ml. 5 per cent sodium hydroxide, dilute to volume, mix, and let stand for 10 minutes. Then add 4 g. powdered potassium chloride, mix by inversion for about 1 minute, filter on a 9-cm. quantitative filter paper, and compare the two filtrates in the colorimeter. If the picrate is pure, the two filtrates will have the same color.

PICRIC ACID. The process for the preparation of pure picric acid is exactly the same as described above, up to the final washing with methyl alcohol, except that hardened filter paper should be used on the Buchner funnel.

One simply converts the purified sodium picrate in the Buchner funnel into picric acid by treating it with dilute hydrochloric acid.

Prepare at least 2 liters of such acid (1 volume conc. acid to 4 volumes water). Disconnect the filtering flask from the suction pump. Pour the acid over the picrate. Stir up the precipitate with a porcelain or glass spoon, so as to make sure that the acid has acted on it all. Use plenty of the acid. Unchanged picrate can be distinguished by its darker color. When no more picrate is visible, connect again with the suction pump, and filter to dryness. Then wash five or six times with cold distilled water and suck as dry as possible. Temperatures up to 90° C. can safely be used for the drying of picric acid.

Picric Acid, Saturated Solution. This may be prepared either by allowing distilled water to stand in contact with an excess of picric acid with occasional shaking, or by making a 1.2 per cent solution.

Potassium Mercuric Iodide. Dissolve 6.775 g. dry crystalline mercuric chloride and 25 g. potassium iodide separately in water. Mix. Dilute to 1000 ml.

Potassium Permanganate Standard (0.1 N) Solution. Dissolve 3.162 g. pure potassium permanganate in a liter of distilled water, allow to stand a few days, and filter through glass wool. Standardize against 0.1 N oxalic acid solution or against pure dry sodium or potassium oxalate. One ml. 0.1 N permanganate is equivalent to 6.7 mg. sodium oxalate. (See also footnote 250, p. 645.)

Potassium Persulfate Solution (Saturated). Add 100 ml. distilled water to 7 g. pure potassium persulfate in a glass-stoppered bottle. Undissolved excess settles and compensates for loss by decomposition.

Ringer's Solution. To 960 ml. 0.154 M NaCl solution add 20 ml. 0.154 M KCl solution and 20 ml. 0.11 M $CaCl_2$ solution.

Roberts' Reagent. Mix 1 volume concentrated nitric acid and 5 volumes saturated solution of magnesium sulfate.

Rosenheim's Iodo-potassium Iodide Solution. Dissolve 2 g. iodine and 6 g. potassium iodide in 100 ml. water. (This is different from Lugol's solution in which the proportion of iodine to potassium iodide is 1:2.)

Sahli's Reagent. This reagent consists of a mixture of equal parts of 48 per cent solution of potassium iodide and 8 per cent solution of potassium iodate.

Schweitzer's Reagent. To 10 parts ammonium hydroxide (sp. gr. 0.90), add 3 parts distilled water. To this mixture, add a slight excess of copper carbonate, shake vigorously and allow to stand over night. Siphon off the clear, supernatant liquid.

Selivanoff's Reagent. Dissolve 0.05 g. resorcinol in 100 ml. dilute (1:2) hydrochloric acid.

Sodium Acetate Solution. Dissolve 100 g. sodium acetate in 800 ml. distilled water, add 100 ml. 30 per cent acetic acid to the solution, and make the volume of the mixture up to 1 liter with distilled water.

Sodium Alcoholate (0.1 N) Solution. The sodium alcoholate is made by dissolving 2.3 g. cleaned metallic sodium in 1 liter absolute alcohol. It may be standardized against pure benzoic acid in washed chloroform, or against 0.1 N HCl provided the alcoholate solution contains not more than traces of carbonate.

Sodium Alizarin Sulfonate. Dissolve 1 g. sodium alizarin sulfonate in 100 ml. water.

Sodium Cobaltinitrite Solution. Prepare according to Kramer and Tisdall as follows:

SOLUTION A. 25 g. cobalt nitrate crystals are dissolved in 50 ml. water and to this solution is added 12.5 ml. glacial acetic acid.

SOLUTION B. 120 g. sodium nitrite (potassium-free, Merck) is dissolved in 180 ml. water. This gives a total volume of about 220 ml. To all of Solution A is added 210 ml. of Solution B. An evolution of nitric oxide gas occurs at once. Air is drawn through the solution until the gas has passed off. The reagent is placed in the ice chest and filtered each time before using. It will keep at least one month.

To 20 ml. of this sodium cobaltinitrite solution add 2 ml. 40 per cent silver nitrate solution. Shake vigorously and filter to remove trace of insoluble precipitate.

Sodium Hydroxide (Saturated Solution). Shake up about 110 g. of best quality NaOH with 100 ml. distilled water in a 300-ml. Erlenmeyer flask ("Pyrex"). Stopper and allow to stand for a couple of days or until the sodium carbonate settles to the bottom leaving a clear solution of NaOH practically free from carbonate, and containing about 75 g. NaOH per 100 ml.

Sodium Hydroxide Standard (0.1 N) Solution. See pp. 867 and 868.

Sodium Thiosulfate Standard (0.1 N) Solution. Weigh out 25 g. ordinary c.p. sodium thiosulfate or 24.83 g. of the pure dry recrystallized salt. Dissolve in water and dilute to a liter. Boiled distilled water must be used. Keep in a bottle with a siphon arrangement and carrying a soda-lime tube to exclude CO_2. It is best standardized against acid potassium iodate $KH(IO_3)_2$. Weigh out accurately 0.3249 g. acid potassium iodate. Dissolve in 50 ml. water, heating gently if necessary. Transfer the solution to a 100-ml. flask, rinsing the beaker carefully and make to mark with water. This solution is exactly decinormal. Pipet 25 ml. into an Erlenmeyer flask, add 1 g. potassium iodide dissolved in a little water, and a few cubic centimeters of dilute hydrochloric acid. Titrate immediately with the thiosulfate solution. When the solution becomes pale yellow add a few ml. 1 per cent solution of soluble starch and titrate to loss of blue color.

Sodium Tungstate Solution. A 10 per cent solution of sodium tungstate in water. The c.p. sodium tungstate made by the J. T. Baker Chemical Co., Phillipsburg, N. J., or by the Mallinckrodt Chemical Co., St. Louis, Mo., is satisfactory. (See also p. 561.) If not easily soluble, prepare a hot 10 per cent solution, cool, and titrate 25 ml., with 10 per cent NaOH and phenolphthalein, to a pink color which lasts for 3 minutes. Add a proportional amount of NaOH to the main solution.

Soluble Starch. Suspend 3 g. raw potato starch in 100 ml. redistilled 95 per cent alcohol, add 0.75 ml. concentrated HCl, and heat on a boiling water bath with a reflux condenser for exactly 10 minutes. Add at once an amount of normal sodium bicarbonate solution just sufficient to neutralize 0.75 ml. of the acid, using methyl orange as indicator. Decant through a filter and wash with several portions of alcohol. Dry at room temperature, sieve and preserve.

Soluble Starch Solution. A solution of soluble starch suitable for most iodometric titrations and with good keeping qualities is made according to Pincussen by dissolving 1 g. soluble starch in 10 ml. boiling water and adding to 90 ml. saturated NaCl solution.

Starch-Iodic Acid Test Paper. This test paper is prepared as follows: Saturate a good quality of filter paper with 0.5 per cent starch paste to which has been added sufficient iodic acid to make a 1 per cent solution of iodic acid and allow the paper to dry in the air. Cut it in strips of suitable size and preserve for use.

Starch Paste. Grind 2 g. starch powder in a mortar with a small amount of water. Bring 200 ml. water to the boiling point and add the starch mixture from the mortar with continuous stirring. Bring again to the boiling point and allow it to cool. This makes an approximate 1 per cent starch paste which is a very satisfactory strength for general use.

Stoke's Reagent. A solution containing 2 per cent ferrous sulfate and 3 per cent tartaric acid. When needed for use a small amount should be placed in a test tube and ammonium hydroxide added until the precipitate which forms on the first addition of the hydroxide has entirely dissolved. This produces *ammonium ferrotartrate*, which is a reducing agent.

Sulfuric Acid, N/12. Add 2.5 ml. concentrated sulfuric acid to a liter of distilled water. Standardize against alkali of known strength. (See p. 544.)

Sulfuric Acid, Two-thirds Normal. Add 35 g. concentrated c.p. sulfuric acid to a liter of distilled water. Standardize against alkali of known strength.

Takayama's Solution. A mixture of 3 ml. 10 per cent NaOH, 3 ml. pyridine, 3 ml. saturated solution of glucose and 7 ml. water. The solution works rapidly in the cold if at least 24 hours old. With a fresh solution, warming or more time is necessary. It keeps for one to two months.

Tannic Acid. A freshly prepared 5 per cent aqueous solution.

Tanret's Reagent. Dissolve 1.35 g. mercuric chloride in 25 ml. water, add to this solution 3.32 g. potassium iodide dissolved in 25 ml. water, then make the total solution up to 60 ml. with distilled water and add 20 ml. glacial acetic acid to the mixture.

Tincture of Iodine. Dissolve 70 g. iodine and 50 g. potassium iodide in 50 ml. distilled water. Dilute to 1000 ml. with 95 per cent alcohol.

Töpfer's Reagent. Dissolve 0.5 g. dimethylaminoazobenzene in 100 ml. 95 per cent alcohol.

Tropeolin 00. Dissolve 0.05 g. tropeolin 00 in 100 ml. 50 per cent alcohol.

Tungstate-Molybdate Reagent. To 10 g. pure, NH_3-free, molybdic acid in a flask add 50 ml. N sodium hydroxide and boil gently for 3 to 5 minutes. Add about 150 ml. water. Filter the hot solution and add to the filtrate a solution of 80 g. sodium tungstate dissolved in about 600 ml. water. Dilute to 1000 ml.

Uranium Acetate Solution. Dissolve about 35.0 g. uranium acetate in 1 liter water with the aid of heat and 3 to 4 ml. glacial acetic acid. Let stand a few days and filter. Standardize against a phosphate solution containing 0.005 g. P_2O_5 per ml. For this purpose dissolve 14.721 g. pure air-dry sodium ammonium phosphate ($NaNH_4HPO_4 + 4H_2O$) in water to make a liter. To 20 ml. of this phosphate solution in a 200-ml. beaker add 30 ml. water and 5 ml. sodium acetate solution (see p. 1332) and titrate with the uranium solution to the correct end reaction as indicated in the method proper, p. 952. If exactly 20 ml. uranium solution are required, 1 ml. of the solution is equivalent to 0.005 g. P_2O_5. If stronger than this, dilute accordingly and check again by titration.

Winkler's Reagent. Cuprous chloride 40 g., ammonium chloride 50 g., distilled water to 150 ml. For use mix this solution with ammonium hydroxide (sp. gr. 0.9) in the proportion of 3:1.

II. COMMON ACIDS AND ALKALIES AS PURCHASED

Substance	*Specific Gravity (at Room Temperature)*	*Per Cent by Weight*	*Approximate Normality*
NH_4OH	0.90	28 (as NH_3)	15
NaOH (saturated)	1.5	50	19
H_2SO_4 (concentrated)	1.84	95	36
HNO_3 (concentrated)	1.42	70	16
HCl (concentrated)	1.19	36	12
H_3PO_4 (syrupy)	1.71	85	45
Acetic acid (glacial)	1.05	99.5	17

III. TABLE OF COMPOSITION OF FOODS*—RAW, PROCESSED, PREPARED

SIGNS AND SYMBOLS USED

An asterisk in the table stub indicates an item for which the composition has been calculated from a recipe.

Parentheses denote imputed values for which little or no experimental evidence was available, for which there was relatively little basis for imputing a value from another form of the food, or for which reported data were not considered suitable. A *zero* in parenthesis is used where actual data were lacking and the amount of a constituent present was regarded as *none or probably too little to measure.*

Dashes show that no basis could be found for imputing a value although there was some reason to believe that a measurable amount of the constituent might be present.

The word "Trace" is used to indicate vitamin values that would round to zero with the number of decimal places carried in these tables. For other components that would round to zero, a zero is used. A zero followed by a decimal point indicates that there may be up to 0.5 of the unit present but bases for showing the amount were inadequate. Numbers with or without decimal points indicate that the average has been rounded to the nearest whole number or in the case of vitamin A to the nearest multiple of ten.

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* Abridged from Watt, Merrill, Orr, Wu, and Pecot: Agriculture Handbook No. 8, Washington, D.C., U.S. Department of Agriculture, 1950.

In preparing this abridgment the following items have been omitted: Raw foods not commonly eaten; certain canned foods when values for corresponding cooked items are included; frozen foods and prepared infant foods similar in composition to forms included; condensed soups; lean or fat meats when medium-fat meats are listed; cross references. Numbers missing from first column represent omitted items.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION

Food and description	Water	Food energy	Protein	Fat	Carbohydrate		Ash	Calcium	Phosphorus	Iron	Vitamin A value	Thiamine	Riboflavin	Niacin	Ascorbic acid
					Total	Fiber									
	Pct.	Cal.	g.	g.	g.	g.	g	mg.	mg.	mg.	I.U.	mg.	mg.	mg.	mg.
1. Almonds, dried, unblanched	4.7	597	18.6	54.1	19.6	2.7	3.0	254	475	4.4	0	0.25	0.67	4.6	Trace
2. Apples, raw	84.1	58	.3	.4	14.9	1.0	.3	6	10	.3	90	.04	.03	.2	5
5. dried, cooked, *unsweetened	78.1	79	.4	.3	20.8	1.1	.4	5	14	.4	(0)	.02	.03	.3	2
7. Apples and apricots, canned, strained (infant food)	82.3	63	.4	.3	16.5	.5	.5	11	16	1.0	1,070	.02	.02	.2	2
8. *Apple betty	64.2	150	1.7	2.9	30.6	.8	.6	15	25	.1	160	.06	.04	.5	1
9. Apple butter	53.1	184	.4	.8	45.4	1.1	.3	14	21	.6	(0)	.01	.02	.2	2
10. Apple juice, fresh or canned	85.9	50	.1	(0)	13.8	—	.3	6	10	.5	40	.02	.03	Trace	1
11. Applesauce, canned; unsweetened	88.4	42	.2	.2	10.9	.6	.3	4	8	.4	30	.02	.01	Trace	1
12. sweetened	79.8	72	.2	.1	19.7	.6	.2	4	8	.4	30	.02	.01	Trace	1
14. Apricots, raw	85.4	51	1.0	.1	12.9	.6	.6	16	23	.5	2,790	.03	.05	.8	7
15. canned, water pack, solids and liquid	90.9	32	.5	.1	8.1	.3	.4	10	15	.3	1,350	.02	.02	.3	4
16. sirup pack, solids and liquid	77.3	80	.6	.1	21.4	.4	.6	10	15	.3	1,350	.02	.02	.3	4
19. dried, sulfured, cooked, *unsweetened, fruit and liquid	75.3	85	1.7	.1	21.8	1.0	1.1	28	39	1.6	2,420	Trace	.05	1.0	3
24. Asparagus, canned, green, solids and liquid	93.6	18	1.9	.3	2.9	.5	1.3	18	43	1.7	600	.07	.10	.9	15
25. drained solids	92.5	21	2.4	.4	3.4	.8	1.3	19	53	1.9	800	.06	.08	1.0	18
26. canned, bleached, solids and liquid	93.3	18	1.6	.3	3.3	.5	1.5	15	33	.9	50	.05	.07	.8	15
27. drained solids	92.3	22	2.1	.5	3.6	.8	1.5	16	41	1.0	80	.05	.08	(.9)	18
29. Avocados, raw[1]	65.4	245	1.7	26.4	5.1	1.8	1.4	10	38	.6	290	.06	.13	1.1	16
30. Bacon, medium fat, raw, slab or sliced	20.	630	9.1	65.	1.1	0	4.3	13	108	.8	(0)	.38	.12	1.9	0
31. broiled or fried, drained	13.	607	25.	55.	1.	0	6.	25	255	3.3	(0)	.48	.31	4.8	0
34. Bananas, raw	74.8	88	1.2	.2	23.	.6	.8	8	28	.6	430	.04	.05	.7	10
35. Barley, pearled, light, dry	11.1	349	8.2	1.0	78.8	.5	.9	16	189	(2.0)	(0)	.12	.08	3.1	0
38. Beans, common or kidney, mature dry seeds; red kidney, canned (or cooked), solids and liquid	76.0	90	5.7	.4	16.4	.9	1.5	40	124	1.9	(0)	.05	.05	.8	0
40. other (including navy, pea bean, white marrow, other), canned, baked, pork and molasses	70.0	125	5.8	3.0	19.2	.9	2.0	56	113	2.1	30	.05	.04	.5	2

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[1] Data on proximate constituents apply to Fuerte variety.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—*(Continued)*

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
41. pork and tomato sauce	71.7	113	5.8	2.1	18.4	1.0	2.0	41	113	1.8	80	0.05	0.04	0.5	2
44. Beans, lima, immature seeds, canned, solids and liquid	80.9	71	3.8	.3	13.5	1.3	1.5	27	73	1.7	130	.04	.04	.5	8
45. drained solids	74.9	95	5.0	.4	18.3	2.0	1.4	29	77	1.7	180	.03	.05	.5	6
51. Beans, snap, green, canned, solids and liquid	93.5	18	1.0	.1	4.2	.6	1.2	27	19	1.4	410	.03	.04	.3	4
52. drained solids	92.5	22	1.4	.2	4.7	.5	1.2	36	23	1.7	500	.04	.05	.4	5
56. wax or yellow, canned, solids and liquid	93.5	18	1.0	.1	4.2	.6	1.2	27	19	1.4	100	.03	.04	.3	4
57. drained solids	92.5	22	1.4	.2	4.7	.5	1.2	36	23	1.7	120	.04	.05	.4	5
59. Beef carcass, raw, side including kidney fat, medium fat	60.	273	17.5	22.	0.	0	.9	10	150	2.6	(0)	.08	.16	4.2	0
62. medium fat carcass, trimmed to retail	63.	240	18.2	18.	0.	0	.9	11	161	2.7	(0)	.08	.16	4.4	0
63. Beef cuts, medium fat, chuck, raw	65.	224	18.6	16.	0.	0	.9	11	167	2.8	(0)	.08	.17	4.5	0
64. cooked	51.	309	26.	22.	0.	0	.7	11	117	3.1	(0)	[2] .05	[2] .20	[2]4.1	0
65. flank, raw	61.	247	19.9	18.	0.	0	.9	12	186	3.0	(0)	.09	.18	4.8	0
66. cooked	51.	314	25.	23.	0.	0	.6	11	117	3.0	(0)	[2] .05	[2] .20	[2]4.1	0
67. hamburger, raw	55.	321	16.	28.	0.	0	.8	9	128	2.4	(0)	.07	.14	3.8	0
68. cooked	47.	364	22.	30.	0.	0	1.1	9	158	2.8	(0)	.08	.19	4.8	0
69. porterhouse, raw	58.	296	16.4	25.	0.	0	.8	10	134	2.5	(0)	.07	.15	3.9	0
70. cooked	49.	342	23.	27.	0.	0	1.1	11	170	3.0	(0)	.06	.18	4.7	0
71. rib roast, raw	59	282	17.4	23.	0.	0	.8	10	149	2.6	(0)	.07	.15	4.2	0
72. cooked	51.	319	24.	24.	0.	0	1.2	10	185	3.0	(0)	.06	.18	4.3	0
73. round, raw	69.	182	19.5	11.	0.	0	1.0	11	180	2.9	(0)	.08	.17	4.7	0
74. cooked	59.	233	27.	13.	0.	0	1.3	11	224	3.4	(0)	.08	.22	5.5	0
75. rump, raw	55.	322	16.2	28.	0.	0	.8	9	131	2.4	(0)	.07	.14	3.9	0
76. cooked	46.	378	21.	32.	0.	0	.5	8	85	2.5	(0)	[2] .04	[2] .15	[2]3.1	0
77. sirloin, raw	62.	254	17.3	20.	0.	0	.9	10	147	2.6	(0)	.07	.15	4.2	0
78. cooked	54.	297	23.	22.	0.	0	1.1	10	175	2.9	(0)	.06	.19	4.8	0
79. Beef, canned, corned beef hash	70.4	141	13.7	6.1	7.2	.2	2.6	26	146	1.3	Trace	.03	.14	2.9	0
80. roast beef	60.	224	25.	13.	0.	0	2.	16	116	2.4	(0)	.02	.23	4.2	0

84. Beef, corned, boneless, canned, medium fat	59.3	216	25.3	12.	0.	0	3.4	20	106	4.3	(0)	0.02	0.24	3.4	0
86. Beef, dried or chipped	47.7	203	34.3	6.3	0.	0	11.6	20	404	5.1	(0)	(.07)	(.32)	(3.8)	0
87. *Beef and vegetable stew	78.6	107	5.5	8.2	7.1	.4	.6	13	75	1.1	1,070	.05	.06	1.5	6
88. Beer (average, 4 per cent alcohol)	90.2	[4]	.6	.0	4.4	—	.2	4	26	.0	(0)	Trace	.03	.2	(0)
91. Beets, common red, canned, solids and liquid	90.3	34	.9	.1	7.9	.5	.8	15	29	.6	20	.01	.02	.1	5
92. drained solids	88.3	41	1.0	.1	9.8	.8	.8	21	31	.7	20	.01	.03	.1	5
95. Beet greens, common, cooked	90.4	27	2.0	.3	5.6	1.4	1.7	[5]118	45	3.2	7,440	.05	.16	.4	15
96. Beverages, carbonated, ginger ale	91.	35	—	—	9.	—	—	—	—	—	—	—	—	—	—
97. other, including kola type	88.	46	—	—	12.	—	—	—	—	—	—	—	—	—	—
98. *Biscuits, baking powder, made with unenriched flour	27.0	342	8.2	10.6	52.2	.2	2.0	218	193	.5	0	.05	.09	.5	0
101. *Biscuits, canned, unbaked	38.6	287	6.9	8.9	43.9	.2	1.7	184	163	1.5	0	.24	.20	1.8	0
102. Blackberries, raw	84.8	57	1.2	1.0	12.5	4.2	.5	32	32	.9	200	.04	.04	.4	21
103. canned, solids and liquids, water pack	88.7	43	.9	.7	9.4	2.0	.3	18	19	(.7)	180	.01	.02	.2	6
104. sirup pack	76.0	86	.7	.2	22.8	2.9	.3	18	19	(.7)	180	.01	.02	.2	6
105. *Blanc mange (vanilla cornstarch pudding)	76.0	111	3.5	3.9	15.7	0.	.9	117	92	.1	160	.03	.16	.1	Trace
106. Blueberries, raw	83.4	61	.6	.6	15.1	1.2	.3	16	13	.8	280	(.02)	(.02)	(.3)	16
107. canned, solids and liquid, water pack	90.	37	.4	.4	9.0	1.0	.2	11	6	(.5)	40	.01	.01	.2	13
108. sirup pack	73.	98	.4	.4	26.	1.0	.2	11	6	(.5)	40	.01	.01	.2	13
111. Bluefish, cooked, baked	69.2	155	27.4	4.2	0.	0	1.9	23	293	.7	—	.12	.11	2.2	—
113. Bouillon cubes	5.	48	(6.)	2.5	(0)	0	68.	—	—	—	—	—	1.8	25.6	0
114. Brains, all kinds, raw	78.9	125	10.4	8.6	.8	0	1.4	16	330	3.6	0	.23	.26	4.4	18
115. Bran (breakfast cereal, almost wholly bran)	2.6	242	12.0	3.4	74.2	8.8	7.8	94	1,312	10.3	(0)	.37	.39	19.2	(0)
116. Bran flakes (40 per cent bran)	3.6	292	10.8	1.9	78.8	3.9	4.9	61	622	5.1	(0)	.46	.23	8.7	(0)
117. Bran, raisin	6.4	297	9.0	1.8	78.6	3.5	4.2	60	541	4.8	(0)	.39	.19	7.0	(0)
118. Brazil nuts	5.3	646	14.4	65.9	11.0	2.1	3.4	186	693	3.4	Trace	.86	—	—	—
119. *Breads, Boston brown bread made with degermed corn meal, unenriched	44.5	219	4.8	2.1	46.0	.3	2.6	185	158	2.5	140	.08	.12	1.4	0
121. cracked-wheat bread, made with unenriched flour	36.0	259	8.5	2.2	51.4	.5	1.9	83	126	1.0	0	.11	.10	1.4	0
123. enriched flour	36.0	259	8.5	2.2	51.4	.5	1.9	83	126	2.0	0	.25	.19	2.5	0
125. French or Vienna breads, unenriched	35.5	270	8.1	2.7	52.0	.2	1.7	24	71	.7	0	.05	.06	.9	0

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[2] Data assume cut to be prepared by braising or pot-roasting. Use of proportionate quantity of drippings would add approximately 50 per cent more thiamine and niacin and 25 per cent more riboflavin.

[3] Data assume cut to be prepared by braising or pot-roasting. Use of proportionate quantity of drippings would add approximately 50 per cent more thiamine and niacin and 25 per cent more riboflavin.

[4] The value excluding energy derived from alcohol is 20 calories. If the energy from alcohol is considered available, the value is 48 calories.

[5] Calcium may not be available because of presence of oxalic acid.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

	Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
						Total	*Fiber*									
		Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
126.	enriched	35.5	270	8.1	2.7	52.0	0.2	1.7	24	71	[6]1.8	0	[6]0.24	[6]0.15	[6]2.2	0
127.	Italian bread, unenriched	35.0	263	8.7	.8	53.7	.2	1.8	13	77	.7	0	.05	.07	1.0	0
128.	enriched	35.0	263	8.7	.8	53.7	.2	1.8	13	77	[6]1.8	0	[6].24	[6].15	[6]2.2	0
129.	raisin bread, unenriched	30.2	284	7.1	3.1	57.8	.2	1.8	80	104	1.3	10	.07	.11	.9	0
131.	enriched	30.2	284	7.1	3.1	57.8	.2	1.8	80	104	[6]1.8	10	[6].24	[6].15	[6]2.2	0
133.	rye bread, American (⅓ rye, ⅔ clear flour)	35.3	244	9.1	1.2	52.4	.4	2.0	72	147	1.6	0	.18	.08	1.5	0
135.	white bread, unenriched, 4 per cent nonfat milk solids[7]	34.7	275	8.5	3.2	51.8	.2	1.8	79	92	.6	0	.05	.11	.9	0
139.	white bread, enriched, 4 per cent nonfat milk solids[7]	34.7	275	8.5	3.2	51.8	.2	1.8	79	92	[6]1.8	0	[6].24	[6].15	[6]2.2	0
140.	toasted	25.5	313	9.7	3.7	59.0	.2	2.1	90	105	2.1	0	.22	.18	2.5	0
142.	whole-wheat bread	36.6	240	9.3	2.6	49.0	1.5	2.5	96	263	2.2	0	.30	.13	3.0	0
145.	Breakfast foods, mixed cereals; corn and soy grits, ready-to-eat (added thiamine and niacin)															
146.	wheat and malted barley, ready-to-eat (added thiamine and niacin)	2.8	389	11.0	.6	82.8	1.8	2.8	47	330	3.5	(0)	.53	.17	4.7	(0)
148.	Broccoli, flower stalks, cooked	89.9	29	3.3	.2	5.5	1.3	1.1	130	76	1.3	3,400	.07	.15	.8	74
151.	Brussels sprouts, cooked	84.9	47	4.4	.5	8.9	1.3	1.3	34	78	1.3	400	.04	.12	.5	47
153.	Buckwheat flour, dark	12.	347	11.7	2.5	72.0	1.6	1.8	33	347	2.8	(0)	.58	.15	2.9	(0)
154.	light	12.	348	6.4	1.2	79.5	.5	.9	11	88	1.0	(0)	.08	(.04)	(.4)	(0)
155.	Butter	15.5	716	.6	81.	.4	0	2.5	20	16	.0	[8]3,300	Trace	.01	.1	0
156.	Buttermilk, cultured (made from skim milk)	90.5	36	3.5	.1	5.1	0	.8	(118)	93	.1	Trace	.04	.18	.1	1
157.	Cabbage, raw	92.4	24	1.4	.2	5.3	1.0	.8	46	31	.5	80	.06	.05	.3	50
158.	cooked	92.4	24	1.4	.2	5.3	1.0	.8	46	31	.5	90	.05	.05	.3	31
162.	Cabbage, celery or chinese, cooked	95.4	14	1.2	.3	2.4	.5	.7	43	41	.9	260	.02	.03	.3	22
163.	*Cakes, angel food	31.6	270	8.4	.3	58.7	0.	1.0	6	24	.3	(0)	.01	.14	.2	(0)
165.	foundation, plain icing	24.1	342	5.0	9.3	60.4	.1	1.2	101	96	.4	[9]120	.02	.07	.2	(0)
167.	fruit, dark	22.9	354	5.2	13.8	55.9	1.2	2.2	97	126	2.8	[9]160	.14	.14	1.1	(0)
[illegible]	[illegible]	26.8	327	6.4	8.2	57.0	.1	1.6	155	137	.4	[9]120	.03	.08	.3	(0)

170.	pound	19.3	434	7.1	23.5	49.3	.1	.8	52	104	1.6	[9]330	.12	.16	.9	(0)
171.	rich	21.6	392	5.0	17.7	54.2	.1	1.5	105	113	.6	[9]210	.03	.08	.2	(0)
173.	sponge	31.8	291	7.9	5.0	54.4	.2	.9	28	110	1.4	520	.05	.15	.2	(0)
174.	Candy, candied or glace peel, citron	18.0	314	.2	.3	80.2	1.4	1.3	83	24	.8	—	—	—	—	—
175.	ginger root, crystallized	12.	340	.3	.2	87.1	.7	.4	—	—	—	—	—	—	—	—
176.	lemon, orange, or grapefruit peel	17.4	316	.4	.3	80.6	2.3	1.3	—	—	—	—	—	—	—	—
177.	*butterscotch	5.0	410	0.	8.9	85.6	0.	.5	20	7	1.8	(0)	(0)	Trace	Trace	(0)
178.	*caramels	7.0	415	2.9	11.6	77.5	0.	1.	126	90	2.3	170	.02	.14	.1	Trace
179.	chocolate, sweetened, milk	1.1	503	(6.)	33.5	55.7	.5	1.7	216	283	4.0	150	.10	.38	.8	(0)
180.	chocolate, sweetened, milk, with almonds	.6	532	(8.)	38.6	50.0	.6	1.8	206	249	2.9	140	.13	.51	(1.1)	(0)
181.	chocolate creams	9.	394	4.	14.	72.	—	1.	—	—	—	—	—	—	—	0
182.	fondant	8.	352	0.	0.	91.	0.	1.	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
183.	*fudge, plain	5.	411	1.7	11.3	81.3	.3	.7	[10]48	67	.3	220	.01	.07	.1	Trace
184.	hard	1.	383	0.	0.	99.	0.	0.	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
185.	marshmallows	15.	325	3.	0.	81.	—	1.	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
186.	*peanut brittle	2.	441	8.3	15.5	72.8	.8	1.3	38	124	2.0	30	.09	.05	4.9	0
187.	Cantaloupes, raw	94.0	20	.6	.2	4.6	.6	.6	17	16	.4	[11]3,420	.05	.04	.5	33
188.	Carrots, raw	88.2	42	1.2	0.3	9.3	1.1	1.0	39	37	.8	12,000	.06	.06	.5	
189.	cooked	91.5	30	.6	.5	6.4	.8	1.0	26	26	.6	12,500	.05	.05	.4	4
194.	Cashew nuts, roasted or cooked	3.6	578	18.5	48.2	27.0	1.3	2.7	46	428	5.0	—	.63	.19	2.1	—
196.	Cauliflower, cooked	91.7	25	2.4	.2	4.9	.9	.8	22	72	1.1	90	.06	.08	.5	28
198.	Celery, bleached, raw	93.7	18	1.3	.2	3.7	.7	1.1	50	40	.5	0	.05	.04	.4	7
200.	Cereal foods (infant food), dry, precooked	5.7	364	14.2	2.4	73.4	1.4	4.3	651	686	33.9	(0)	1.19	.46	[12]4.9	(0)
202.	Chard, leaves and stalks, cooked	91.8	21	1.4	.2	4.4	.9	2.2	[13]105	36	2.5	3,110	.04	.06	.4	17
204.	Chard, leaves only, cooked	91.	27	2.6	.4	4.8	.8	1.2	[13]105	36	2.5	9,690	.04	.16	.3	17
205.	Cheese, blue mold, domestic type	40.	368	21.5	30.5	2.0	0	6.0	315	339	(.5)	(1,240)	.03	.61	.4	(0)
206.	Camembert	52.2	299	17.5	24.7	1.8	0	3.8	105	184	.5	(1,020)	.04	.75	1.1	(0)
207.	Cheddar	37.	398	25.0	32.2	2.1	0	3.7	725	495	1.0	1,400	.02	.42	Trace	(0)

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[6] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in standards of identity proposed by the Federal Security Agency and published in the Federal Register, August 3, 1943.

[7] When the amount of nonfat milk solids in commercial bread is unknown, use bread with 4 per cent nonfat milk solids, item 135 for unenriched bread and 139 for enriched.

[8] Year-round average.

[9] If the fat used in the recipe is butter or fortified margarine, the vitamin A value per 100 g. would be 540 I.U. in foundation cake; 430 I.U. in foundation cake, iced; 410 I.U. in dark fruit cake; 370 I.U. in plain cake; 280 I.U. in plain cake, iced; 990 I.U. in pound cake; 830 I.U. in rich cake; and 690 I.U. in rich cake, iced.

[10] If the calcium contributed by chocolate is considered unavailable, the value would be 38 mg. per 100 g.

[11] Vitamin A based on deeply colored varieties.

[12] Based on products ranging from 2.5 to 6.6 mg. per 100 g. cereal. The niacin value of some products is as high as 23.0 mg.

[13] Calcium may not be available because of presence of oxalic acid.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
208. Cheddar, processed	40.	370	23.2	29.9	2.0	0	4.9	673	[14]787	0.9	(1,300)	.02	0.41	Trace	(0)
210. cottage, from skim milk	76.5	95	19.5	.5	2.0	0	1.5	96	189	.3	(20)	.02	.31	(.1)	(0)
211. cream cheese	51.	371	9.0	37.0	2.0	0	1.0	68	97	.2	(1,450)	(.01)	.22	.1	(0)
212. Limburger	45.	345	21.2	28.0	2.2	0	3.6	590	393	.6	1,280	.08	.50	.2	(0)
213. Parmesan	30.	393	36.0	26.0	2.9	0	5.1	1,160	823	.4	(1,060)	.02	.73	.2	(0)
214. Swiss	39.	370	27.5	28.0	1.7	0	3.8	925	563	.9	1,450	.01	(.40)	(.1)	(0)
216. Cherries, sour, sweet, and hybrid, raw	83.0	61	1.1	.5	14.8	.3	.6	18	20	.4	620	.05	.06	.4	8
217. Cherries, red, sour, pitted, canned	86.6	48	.8	.3	11.9	.1	.4	11	12	(.3)	720	.03	.02	.2	6
218. Chicken,[15] raw, broilers, total edible	71.2	151	20.2	7.2	0.	0	1.1	14	200	1.5	(0)	.08	.16	10.2	(0)
219. roasters, total edible	66.0	200	20.2	12.6	0.	0	1.0	14	200	1.5	(0)	.08	.16	8.0	(0)
220. hens, total edible	55.9	302	18.0	25.0	0.	0	1.1	14	200	1.5	(0)	.08	.16	8.0	(0)
221. fryers (cut-up pieces), breast	74.9	104	23.3	.5	0.	0	1.2	14	212	1.1	(0)	.07	.09	10.5	(0)
222. leg	74.5	112	20.5	2.7	0.	0	1.1	15	188	1.8	(0)	.10	.24	5.6	(0)
223. canned, boned, meat only	61.9	199	29.8	8.0	0.	0	2.4	14	148	1.8	(0)	.04	.16	6.4	(0)
225. Chile con carne (without beans), canned[16]	66.9	200	10.3	14.8	5.8	.2	2.2	38	152	1.4	150	.02	.12	2.2	—
226. Chili sauce	68.7	98	2.8	.4	23.7	.7	4.4	(12)	(18)	(.8)	(1,880)	(.09)	(.07)	(2.2)	(11)
227. Chocolate, bitter or unsweetened	2.3	501	(5.5)	52.9	[17]29.2	2.6	3.2	[18]98	446	(4.4)	60	.05	.24	1.1	(0)
228. sweetened, plain	1.4	471	(2.)	29.8	62.7	1.4	1.4	[18](63)	(287)	2.8	(30)	(.03)	(.15)	(.6)	(0)
230. Chocolate sirup	39.0	209	(1.2)	1.1	56.6	.6	.6	[18](15)	(86)	(1.4)	—	—	—	—	—
231. Clams, long and round, raw, meat only	80.3	81	12.8	1.4	3.4	—	2.1	(96)	(139)	(7.0)	110	.10	.18	(1.6)	—
233. Cocoa, breakfast, plain, dry powder	3.9	293	(8.)	23.8	[17]48.9	4.6	5.0	[18]125	712	11.6	(30)	.12	.38	2.3	(0)
234. *Cocoa beverage, made with all milk	79.0	95	3.8	4.6	10.9	.1	.9	119	114	.4	160	.04	.19	.2	1
235. Coconut, fresh, meat	46.9	359	3.4	34.7	14.0	3.2	1.0	21	98	2.0	0	.10	.01	.2	2
236. dried, shredded (sweetened)	3.3	556	3.6	39.1	53.2	4.1	.8	43	191	3.6	0	Trace	Trace	Trace	(0)
239. Cod, dried	12.3	375	81.8	2.8	0.	0	7.0	(50)	891	3.6	0	.08	.45	10.9	(0)
240. *Coleslaw	83.6	86	1.3	6.1	7.7	.9	1.3	39	27	.4	70	.05	.04	.2	41
241. Collards, raw	86.6	40	3.9	.6	7.2	1.2	1.7	249	58	1.6	6,870	.11	.27	(2.0)	100
242. cooked (in small or moderate amount of water)	86.6	40	3.9	.6	7.2	1.2	1.7	249	58	1.6	7,630	.08	.24	(1.7)	44
Condensed milk. See Milk, cow															

244. Cookies, plain and assorted	4.8	436	6.0	12.7	75.0	—	1.6	22	65	.6	(0)	.04	.04	.5	(0)
246. Corn, sweet, white or yellow, cooked	75.5	85	2.7	.7	20.2	—	.9	5	52	.6	[19]390	.11	.10	1.4	8
250. *Corn bread or muffins, made with whole ground corn meal	49.2	215	7.2	5.7	34.8	.6	3.1	141	216	1.7	[22]130	.15	.18	.8	(0)
251. enriched, degermed corn meal	49.2	219	6.7	4.7	36.6	.2	2.8	139	155	1.9	[21]130	.17	.23	1.3	(0)
253. Corn flakes, (enriched)	3.6	385	8.1	.4	85.0	.6	2.9	11	58	2.2	(0)	.41	.10	2.2	(0)
254. Corn flour	12.	368	7.8	2.6	76.8	.7	.8	6	(178)	1.8	[22]340	.20	.06	(1.4)	(0)
255. Corn grits, degermed, unenriched, dry	12.	362	8.7	.8	78.1	.4	.4	4	73	1.0	[23]300	.13	.04	1.2	(0)
256. *cooked	87.1	51	1.2	.1	11.0	.1	.6	1	10	.1	[23]40	.02	.01	.2	(0)
257. enriched, dry	12.	362	8.7	.8	78.1	.4	.4	4	73	[24]2.9	[23]300	[24].44	[24].26	[24]3.5	(0)
260. Corn meal, white or yellow, whole ground, dry, bolted	12.	362	9.0	3.4	74.5	1.0	1.1	6	(178)	1.8	[25]440	.30	.08	1.9	(0)
261. degermed, unenriched, dry	12.	363	7.9	1.2	78.4	.6	.5	6	99	1.1	[25]300	.14	.05	1.0	(0)
263. degermed, enriched, dry	12.	363	7.9	1.2	78.4	.6	.5	6	99	[24]2.9	[25]300	[24].44	[24].26	[24]3.5	(0)
265. self-rising, dry, unenriched	12.	340	8.7	3.7	70.8	1.5	4.8	262	634	2.3	[25]480	.36	.10	1.9	(0)
266. enriched	12.	340	8.7	3.7	70.8	1.5	4.8	262	634	[24]2.9	[25]480	[24].44	[24].26	[24]3.5	(0)
268. Cowpeas, immature seeds, cooked	74.9	94	7.1	.6	15.9	—	1.5	37	182	2.5	390	.29	.08	.8	20
271. Crabs, Atlantic and Pacific, hard shell, raw	80.0	86	16.1	1.6	.6	—	1.7	(39)	(160)	(.8)	—	.14	.06	2.7	—
272. canned or cooked, meat only	77.2	104	16.9	2.9	1.3	—	1.7	45	182	.9	—	(.05)	(.06)	(2.5)	—
273. Crackers, graham	5.5	393	8.0	10.0	74.3	.8	2.2	20	203	1.9	(0)	.30	.12	1.5	(0)
274. saltines	4.6	431	9.2	11.8	71.1	.4	3.3	19	92	1.0	(0)	.06	.04	1.0	(0)
275. soda, plain	5.7	420	9.6	9.6	72.7	.2	2.4	20	96	1.1	(0)	.06	.05	1.1	(0)
278. Cranberry sauce, sweetened, canned or cooked	48.1	198	.1	.3	51.4	.4	.1	(8)	(7)	(.3)	(30)	(.02)	(.02)	(.1)	2
279. Cream, light, table or coffee	72.5	204	2.9	20.0	4.0	0	.6	97	77	.1	830	.03	.14	.1	1
280. heavy or whipping	59.	330	2.3	35.0	3.2	0	.5	78	61	.0	1,440	.02	.11	.1	1

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[14] 460 mg. if the added emulsifying agent does not contain phosphorus.

[15] Vitamin values are based on muscle meat only.

[16] Not less than 60 per cent meat, not more than 8 per cent cereals, seasonings.

[17] Approximately one-third of this total amount of carbohydrate calculated by difference is starch and sugar. The remaining portion is made up of materials thought to be utilized only poorly if at all by the body.

[18] Calcium may not be available because of presence of oxalic acid.

[19] Vitamin A based on yellow corn; white corn contains only a trace.

[20] Based on recipe using white corn meal; if yellow corn meal is used the vitamin A value is 330 I.U.

[21] Based on recipe using white corn meal; if yellow corn meal is used the vitamin A value is 250 I.U.

[22] Vitamin A based on yellow corn flour; white corn flour contains only a trace.

[23] Vitamin A based on yellow corn grits; white corn grits contain only a trace.

[24] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in standards of identity promulgated under the Food, Drug, and Cosmetic Act.

[25] Vitamin A based on yellow corn meal; white corn meal contains only a trace.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
281. Cress, garden, raw	87.2	41	4.2	1.4	5.3	1.2	1.9	211	(38)	(2.9)	2,970	0.11	0.17	1.0	87
282. cooked (in small or moderate amount of water)	87.2	41	4.2	1.4	5.3	1.2	1.9	211	(38)	(2.9)	3,300	.07	.15	.8	39
284. Cress, water, leaves and stems, raw	93.6	18	1.7	.3	3.3	.5	1.1	195	46	2.0	4,720	.08	.16	.8	77
286. Cucumbers, raw	96.1	12	.7	.1	2.7	.5	.4	10	21	[28].3	[26]0	.03	.04	.2	8
287. Currants, red, raw	84.4	55	1.2	.2	13.6	4.0	.6	36	33	.9	120	.04	—	—	36
288. *Custard, baked	77.3	114	5.3	5.4	11.2	0	.8	114	119	.5	340	.05	.20	.1	Trace
291. Dandelion greens, cooked	85.8	44	2.7	.7	8.8	1.8	2.0	187	70	3.1	15,170	.13	.12	(.7)	16
292. Dates, "fresh" and dried	20.	284	2.2	.6	75.4	2.4	1.8	72	60	2.1	60	.09	.10	2.2	(0)
293. Doughnuts, cake type	18.7	425	6.6	21.0	52.7	.2	1.0	73	286	(.7)	140	.16	.13	1.2	(0)
295. Eggplant, raw	92.7	24	1.1	.2	5.5	.9	.5	15	37	.4	30	.04	.05	.6	5
296. Eggs, hen, fresh, stored, or frozen, raw, whole	74.0	162	12.8	11.5	.7	0	1.0	54	210	2.7	1,140	.10	.29	.1	0
297. white	87.8	50	10.8	0.	.8	0	.6	6	17	.2	(0)	0	.26	(.1)	0
298. yolk	49.4	361	16.3	31.9	.7	0	1.7	147	586	7.2	3,210	.27	.35	Trace	0
299. hard-cooked	74.0	162	12.8	11.5	.7	0	1.0	54	210	2.7	1,140	.08	.27	.1	0
303. dried, whole	5.	592	46.8	42.0	2.5	0	3.6	190	767	8.8	3,740	.34	1.06	.2	0
304. white	3.	398	85.9	0.	6.3	0	4.8	48	135	1.6	0	0	2.05	.7	0
305. yolk	3.	693	31.2	61.2	1.3	0	3.3	282	1,123	13.8	5,540	.50	.66	.1	0
306. Endive, raw	93.3	20	1.6	.2	4.0	.8	.9	79	56	1.7	3,000	.07	.12	.4	11
307. Farina, unenriched, raw	10.5	370	10.9	.8	77.4	.4	.4	28	112	1.0	(0)	.06	.06	.8	(0)
309. enriched, raw	10.5	370	10.9	.8	77.4	.4	.4	28	112	[27]1.3	(0)	[27].37	[27].26	[27]1.3	(0)
310. *cooked	89.2	44	1.3	.1	9.1	.0	.3	3	13	.2	0	.04	.03	.2	(0)
311. Fats, cooking (vegetable fat)	0.	884	0.	100.	0.	0	0	0	0	0	0	0	0	0	0
312. Figs, raw	78.0	79	1.4	.4	19.6	1.7	.6	54	32	.6	80	.06	.05	.5	2
313. canned, sirup pack, solids and liquid	68.5	113	.8	.3	30.0	.9	.4	35	21	.4	50	.03	.03	.4	Trace
314. dried	24.	270	4.0	1.2	68.4	5.8	2.4	186	111	3.0	80	.16	.12	1.7	(0)
315. Fig bars	13.8	350	4.2	4.8	75.8	1.7	1.4	69	69	1.3	0	.02	.06	.9	(0)
316. Flounder, summer and winter, raw	82.7	68	14.9	.5	0	0	1.3	61	195	.8	—	.06	.05	1.7	—
317. Frog legs, raw	81.9	73	16.4	.3	0.	0	1.1	18	147	1.1	0	.14	.25	1.2	—
318. Fruit cocktail, canned, solids and liquids	80.6	70	.4	.2	18.6	.4	.3	9	12	.4	160	.01	.01	.4	2

319. Gelatin, dry, plain	13.0	335	85.6	.1	0.	0	1.3	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
320. dessert powder	1.6	380	9.4	.0	88.7	0	.3	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
321. *Gelatin dessert, ready-to-serve, plain	83.1	65	1.6	.0	15.2	0	.1	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
323. *Gingerbread	30.4	327	3.9	12.0	51.6	.1	2.1	114	71	2.5	100	.04	.08	1.0	(0)
325. Grapefruit, raw	88.8	40	.5	.2	10.1	.3	.4	22	18	.2	Trace	.04	.02	.2	40
326. canned in sirup, solids and liquid	79.8	72	.6	.2	19.1	.2	.4	13	14	.3	Trace	.03	.02	.2	30
328. Grapefruit juice, canned, unsweetened	89.2	38	.5	.1	9.8	.1	.4	8	13	.3	Trace	.03	.02	.2	35
329. sweetened	85.3	52	.5	.1	13.7	.1	.4	8	13	.3	Trace	.03	.02	.2	35
334. Grapes, raw, American type (slip skin) as Concord, Delaware, Niagara, and Scuppernong	81.9	70	1.4	1.4	14.9	.5	.4	17	21	.6	80	.06	.04	.2	4
335. European type (adherent skin) as Malaga, Muscat, Sultanina (Thompson Seedless), and Flame Tokay	81.6	66	.8	.4	16.7	.5	.5	17	21	.6	80	.06	.04	.2	4
336. Grape juice, bottled, commercial	81.	67	.4	.0	18.2	—	.4	10	10	.3	—	.04	.05	(.2)	Trace
337. Guavas, common, raw	80.6	70	1.0	.6	17.1	5.5	.7	30	29	.7	250	.07	.04	1.2	302
339. Haddock, cooked, fried	66.9	158	18.7	5.5	7.0	—	1.9	18	182	.6	—	.04	.09	2.6	—
341. Halibut, cooked, broiled	64.2	182	26.2	7.8	0.	0	1.9	14	267	.8	—	.06	.07	10.5	—
342. Heart, beef, lean, raw	77.6	108	16.9	3.7	.7	0	1.1	9	203	4.6	30	.58	.89	7.8	6
345. pork, raw	76.8	117	16.9	4.8	.4	0	1.1	35	132	2.7	30	.43	1.24	6.0	6
346. Herring, Atlantic, raw	67.2	191	18.3	12.5	0.	0	2.7	—	256	1.1	110	.02	.15	3.4	—
347. Herring, lake, raw	74.0	140	18.5	6.8	0.	0	1.1	12	152	.5	(100)	.09	.09	3.1	—
348. Herring, Pacific, raw	79.6	94	16.6	2.6	0.	0	1.3	—	—	—	100	.02	.22	(2.2)	—
349. Herring, smoked, kippered	61.0	211	22.2	12.9	0.	0	4.0	66	254	(1.4)	0	Trace	.28	(2.9)	—
350. Honey, strained or extracted	20.	294	.3	0.	79.5	—	.2	5	16	.9	(0)	Trace	.04	.2	4
351. Honeydew melon, raw	90.5	32	.5	0.	8.5	.4	.5	(17)	(16)	(.4)	40	.05	.03	.2	23
352. Ice cream, plain	62.1	207	4.0	12.5	20.6	0	.8	123	99	.1	520	.04	.19	.1	1
353. Jams, marmalades, preserves	28.	278	.5	.3	70.8	.6	.4	12	12	.3	10	.02	.02	.2	6
354. Jellies	34.5	252	.2	.0	65.0	0	.3	(12)	(12)	(.3)	(10)	(.02)	(.02)	(.2)	4
356. Kale, cooked	86.6	40	3.9	.6	7.2	1.2	1.7	225	62	2.2	8,380	.07	.23	1.7	51
358. Kidneys, raw, beef	74.9	141	15.0	8.1	.9	0	1.1	9	221	7.9	1,150	.37	2.55	6.4	13
359. pork	77.1	114	16.3	4.6	.8	0	1.2	11	246	8.0	130	.58	1.74	9.8	13
360. sheep	77.8	105	16.6	3.3	1.0	0	1.3	13	237	9.2	(1,150)	.51	2.42	7.4	13
362. Kohlrabi, cooked	90.1	30	2.1	.1	6.7	1.1	1.0	46	50	.6	Trace	.04	.04	.2	37
364. Lamb, carcass or side, raw, medium fat	55.8	317	15.7	27.7	0.	0	.8	9	157	2.4	(0)	.14	.20	4.5	0
367. retail items:[28] rib chop, medium fat, cooked	40.	418	24.	35.	0.	0	1.2	11	200	3.0	(0)	.14	.26	5.6	0

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[26] Based on pared cucumbers; unpared contain about 1.2 mg. iron and 260 I.U. vitamin A per 100 g.

[27] Iron, thiamine, riboflavin, and niacin are based on the minimum levels of enrichment specified in standards of identity promulgated under the Food, Drug, and Cosmetic Act.

[28] Values for raw items are from the medium fat wholesale cuts considered to be nearest approximations for indicated retail items.

Composition of Foods, 100 G., Edible Portion—(*Continued*)

Food and description	Water	Food energy	Protein	Fat	Carbohydrate		Ash	Calcium	Phosphorus	Iron	Vitamin A value	Thiamine	Riboflavin	Niacin	Ascorbic acid
					Total	Fiber									
	Pct.	Cal.	g.	g.	g.	g.	g.	mg.	mg.	mg.	I.U.	mg.	mg.	mg.	mg.
369. shoulder roast (wholesale 3-rib), cooked	50.	342	21.	28.	0.	0	1.0	9	188	2.6	(0)	0.12	0.22	4.6	0
371. leg roast (wholesale leg), cooked	56.	274	24.	19.	0.	0	1.1	10	257	3.1	(0)	.14	.25	5.1	0
373. Lard	0.	902	0.	100.	0.	0	0	0	0	0	0	0	0	0	0
374. Lemons	89.3	32	.9	.6	8.7	.9	.5	40	22	.6	0	.04	Trace	.1	50
375. Lemon juice, fresh	91.4	24	.4	.2	7.7	.0	.3	14	11	.1	0	.04	Trace	.1	50
378. Lentils, dry, whole (entire seeds)	11.2	337	25.0	1.0	59.5	3.7	3.3	59	423	7.4	570	.56	.24	2.2	5
379. split (without seed coat)	12.2	339	24.0	1.2	60.4	1.7	2.2	34	292	7.4	570	.56	.24	2.2	5
380. Lettuce, raw, headed	94.8	15	1.2	.2	2.9	.6	.9	22	25	.5	540	.04	.08	.2	8
381. all other	94.8	15	1.2	.2	2.9	.6	.9	62	20	1.1	1,620	.04	.08	.2	18
382. Limes	86.0	37	.8	.1	12.3	(.9)	.8	(40)	(22)	(.6)	0	(.04)	(Trace)	(.1)	27
383. Lime juice, fresh	91.0	24	.4	.0	8.3	.0	.3	(14)	(11)	(.1)	0	(.04)	(Trace)	(.1)	27
384. Liver, beef, raw	69.7	136	19.7	3.2	6.0	0	1.4	7	358	6.6	43,900	.26	3.33	13.7	31
385. cooked, fried	57.2	208	23.6	7.7	9.7	0	1.8	8	486	7.8	53,500	.26	3.96	14.8	31
386. calf, raw	70.8	141	19.0	4.9	4.0	0	1.3	6	343	10.6	22,500	.21	3.12	16.1	36
387. chicken, raw	69.6	141	22.1	4.0	2.6	0	1.7	16	240	7.4	32,200	.20	2.46	11.8	20
388. pork, raw	72.3	134	19.7	4.8	1.7	0	1.5	10	362	18.0	14,200	.40	2.98	16.7	23
389. sheep or lamb, raw	70.8	136	21.0	3.9	2.9	0	1.4	8	364	12.6	50,500	.40	3.28	16.9	33
391. Lobster, raw	79.2	88	16.2	1.9	.5	0	2.2	61	184	.6	—	(.13)	.06	(1.9)	—
393. Loganberries, raw	82.9	62	1.0	.6	15.0	1.4	.5	35	19	1.2	(200)	(.03)	(.07)	(.3)	24
395. Macaroni, unenriched, *cooked	60.6	149	5.1	.6	30.2	.2	3.5	9	65	.6	(0)	.02	.02	.5	(0)
397. *enriched, cooked	60.6	149	5.1	.6	30.2	.2	3.5	9	65	[29]1.1	(0)	[29].17	[29].10	[29]1.4	(0)
399. Mackerel, raw, common Atlantic	68.1	188	18.7	12.	0.	0	1.2	5	239	1.0	(450)	.15	.35	8.4	—
400. canned, solids and liquid,[30] Atlantic	66.0	182	19.3	11.1	0.	0	3.2	185	274	2.1	430	.06	.21	5.8	—
401. Pacific	66.4	180	21.1	10.0	0.	0	2.5	260	288	2.2	30	.03	.33	8.8	—
402. Mangos, raw	81.4	66	.7	.2	17.2	1.0	.5	9	13	.2	6,350	.06	.06	.9	41
403. Margarine	15.5	720	.6	81.	.4	0	2.5	20	16	0	[31]3,300	(0)	(0)	(0)	(0)
404. Milk, cow's, fluid (pasteurized and raw), whole	87.0	68	3.5	3.9	4.9	0	.7	118	93	.1	(160)	.04	.17	.1	1
405. nonfat (skim)	90.5	36	3.5	.1	5.1	0	.8	123	97	.1	Trace	.04	.18	.1	1
406. canned, evaporated (unsweetened)	73.7	138	7.0	7.9	9.9	0	1.5	243	195	.2	400	.05	.36	.2	1

407. condensed (sweetened)	27.0	320	8.1	8.4	54.8	0	1.7	273	228	.2	(430)	.05	.39	.2	1
408. dried, whole	3.5	492	25.8	26.7	38.0	0	6.0	949	728	.6	1,400	.30	1.46	.7	6
409. nonfat solids (skim)	3.5	362	35.6	1.0	52.0	0	7.9	1,300	1,030	.6	(40)	.35	1.96	1.1	7
410. malted,[32] dry powder	2.6	407	14.6	8.5	70.7	.3	3.6	287	379	2.1	1,020	.33	.54	—	(0)
411. *beverage	78.2	104	4.6	4.4	11.8	.0	1.0	135	123	.3	250	.07	.21	—	1
412. *chocolate flavored	83.0	74	3.2	2.2	10.6	(0)	.8	109	91	.1	90	.03	.16	.1	1
413. half-and-half (milk and cream)	79.7	137	3.2	12.0	4.5	0	.6	108	85	.1	490	.03	.16	.1	1
414. Milk, goat, fluid	87.4	67	3.3	4.0	4.6	0	.7	129	106	.1	(160)	.04	.11	.3	1
415. Molasses, cane, first extraction or light	24.	252	—	—	[33]65.	—	[34]6.3	165	45	4.3	—	.07	.06	.2	—
417. third extraction or blackstrap	24.	213	—	—	[33]55.	—	[34]10.5	579	85	11.3	—	.28	.25	2.1	—
418. Barbados	24.	271	—	—	[33]70.	—	[34]1.6	—	50	—	—	.06	.20	—	—
419. *Muffins, made with: unenriched flour	37.4	280	8.0	8.4	42.1	.1	2.2	206	191	.6	100	.05	.13	.4	(0)
420. enriched flour	37.4	280	8.0	8.4	42.1	.1	2.2	206	191	1.6	100	.18	.21	1.5	(0)
421. Mung bean sprouts, raw	92.4	23	2.9	.2	4.1	.7	.4	29	59	.8	10	.07	.09	.5	15
422. Mushrooms, raw	91.1	16	2.4	.3	4.0	.9	1.1	9	115	1.0	0	.10	.44	4.9	5
423. canned, solids and liquid	93.0	11	1.4	.2	3.7	—	1.0	(7)	(90)	(.8)	0	.02	.25	2.0	—
425. Mustard greens, cooked	92.2	22	2.3	.3	4.0	.8	1.2	220	38	2.9	7,180	.06	.18	.7	45
427. Noodles (containing egg), unenriched, *cooked	83.8	67	2.2	.6	12.8	.1	.6	4	35	.4	30	.03	.02	.4	(0)
429. enriched, *cooked	83.8	67	2.2	.6	12.8	.1	.6	4	35	[35].5	30	[35].14	[35].06	[35]1.0	(0)
431. Oatmeal or rolled oats, dry	8.3	390	14.2	7.4	68.2	1.2	1.9	53	405	4.5	(0)	.60	.14	1.0	(0)
432. *cooked	84.8	63	2.3	1.2	11.0	.2	.7	9	67	.7	(0)	.10	.02	.2	(0)
434. Oils, salad or cooking	0.	884	0.	100.	0.	0	0	0	0	0	0	0	0	0	0
436. Okra, cooked	89.8	32	1.8	.2	7.4	1.0	.8	82	62	.7	740	.06	.06	.8	20
437. Olives, pickled, green	75.2	132	1.5	13.5	4.0	1.2	5.8	87	17	1.6	300	Trace	—	—	—
438. ripe, mission	71.8	191	1.8	21.0	2.6	1.5	2.8	87	17	1.6	60	Trace	Trace	—	—
439. other varieties (as ascalano, manzanilla, and sevilano)	79.8	128	1.2	13.5	3.1	1.7	2.4	87	17	1.6	60	Trace	Trace	—	—
440. Onions, mature, raw	87.5	45	1.4	.2	10.3	.8	.6	32	44	.5	50	.03	.04	.2	9
441. cooked	89.5	38	1.0	.2	8.7	.8	.6	32	44	.5	50	.02	.03	.2	6

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[29] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in the standards of identity promulgated under the Food, Drug, and Cosmetic Act.

[30] The vitamin values are based on the drained solids.

[31] Based on the average vitamin A content of fortified margarine. Most of the margarines manufactured for use in the United States have 15,000 I.U. of vitamin A added per pound. The minimum Federal specifications for fortified margarine require the addition of 9,000 I.U. of vitamin A per pound.

[32] Based on unfortified products.

[33] Total sugars.

[34] Sulfated ash. This overestimates the ash in the range of 8 to 20 per cent.

[35] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in the standards of identity promulgated under the Food, Drug, and Cosmetic Act.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
442. dehydrated, flaked	4.	347	10.8	1.1	80.2	4.5	3.9	168	273	3.4	130	0.25	0.18	1.4	36
443. Onions, young, green	87.6	45	1.0	.2	10.6	1.8	.6	135	24	.9	(50)	(.03)	(.04)	(.2)	24
444. Oranges	87.2	45	.9	.2	11.2	.6	.5	33	23	.4	(190)	.08	.03	.2	49
445. Orange juice, fresh	87.5	44	.8	.2	11.0	.1	.4	19	16	.2	(190)	.08	.03	.2	49
446. canned, unsweetened	87.5	44	.8	.2	11.1	.1	.4	10	18	.3	(100)	.07	.02	.2	42
447. sweetened	84.8	54	.6	.2	13.9	.1	.5	10	18	.3	(100)	.07	.02	.2	42
449. Orange juice concentrate, frozen	58.	149	2.7	.7	37.1	.2	1.5	34	60	1.0	(330)	.24	.05	.7	141
450. Oysters, meat only, raw	80.5	84	9.8	2.1	5.6	—	2.0	94	143	5.6	320	.15	.20	1.2	—
453. *Pancakes (griddlecakes), baked, wheat (home recipe), with unenriched flour	55.4	218	6.8	9.2	26.6	.1	2.0	158	154	.6	200	.06	.13	.3	Trace
454. with enriched flour	55.4	218	6.8	9.2	26.6	.1	2.0	158	154	1.3	200	.18	.21	1.3	Trace
455. buckwheat, with buckwheat pancake mix	62.0	176	6.1	8.4	20.9	.5	2.6	249	362	1.2	110	.16	.16	.9	Trace
456. Pancake mix, dry, self-rising, wheat (mixed with other flours), unenriched	10.4	349	9.5	1.4	73.1	.4	5.6	465	681	2.0	0	.14	.07	1.4	(0)
457. enriched	10.4	349	9.5	1.4	73.1	.4	5.6	465	681	3.3	0	.39	.31	2.9	(0)
458. buckwheat	11.2	319	10.5	1.9	70.3	1.4	6.1	467	827	3.1	0	.37	.11	2.2	(0)
459. Papayas, raw	88.7	39	.6	.1	10.0	.9	.6	20	16	.3	1,750	.03	.04	.3	56
460. Parsley, common, raw	83.9	50	3.7	1.0	9.0	1.8	2.4	[33]193	84	4.3	8,230	.11	.28	1.4	193
462. Parsnips, cooked	83.5	60	1.0	.5	13.9	2.1	1.1	57	80	.7	0	.06	.10	.2	12
463. Peaches, raw	86.9	46	.5	.1	12.0	.6	.5	8	22	.6	880	.02	.05	.9	8
464. canned, solids and liquid, water pack	92.3	27	.5	.1	6.8	.3	.3	5	14	.4	450	.01	.02	.7	4
465. sirup pack	80.9	68	.4	.1	18.2	.4	.4	5	14	.4	450	.01	.02	.7	4
469. dried, sulfured, *cooked, no sugar added	76.2	83	.9	.2	21.8	1.1	.9	14	39	2.2	1,020	Trace	.06	1.6	4
471. Peanuts, Virginia type, roasted, shelled	2.6	559	26.9	44.2	23.6	2.4	2.7	74	393	1.9	0	.30	.13	16.2	(0)
472. Peanut butter	1.7	576	26.1	47.8	21.0	2.0	3.4	74	393	1.9	0	.12	.13	16.2	(0)
473. Pears, raw	82.7	63	.7	.4	15.8	1.4	.4	13	16	.3	20	.02	.04	.1	4
474. canned, solids and liquid, water pack	91.2	31	.3	.1	8.2	.7	.2	8	10	.2	Trace	.01	.02	.1	2
475. sirup pack	81.1	68	.2	.1	18.4	.8	.2	8	10	.2	Trace	.01	.02	.1	2
477. Peas, green, immature, raw	74.3	98	6.7	.4	17.7	2.2	.9	22	122	1.9	680	.34	.16	2.7	26

478. cooked	81.7	70	4.9	.4	12.1	2.2	.9	22	122	1.9	720	.25	.14	2.3	15
480. canned, drained solids	76.7	91	4.5	.6	17.2	2.3	1.0	32	77	2.1	670	.12	.06	1.0	9
483. Peas, mature dry seeds, entire seeds	11.6	339	23.8	1.4	60.2	5.4	3.0	57	388	4.7	370	.77	.28	3.1	2
484. split, without seed coat	10.0	344	24.5	1.0	61.7	1.2	2.8	33	268	5.1	370	.77	.28	3.1	2
485. Pecans	3.0	696	9.4	73.0	13.0	2.2	1.6	74	324	2.4	50	.72	.11	.9	2
486. Peppers, green, raw	92.4	25	1.2	.2	5.7	1.4	.5	11	25	.4	630	.04	.07	.4	120
487. cooked, parboiled then baked	91.9	26	1.3	.2	6.0	1.6	.6	11	25	.4	740	.04	.07	.4	99
488. Persimmons, Japanese or Kaki, raw	78.2	78	.8	.4	20.0	1.9	.6	6	26	.3	2,710	.05	.05	Trace	11
489. Pickles, dill, cucumber	93.2	11	.7	.2	2.1	.4	3.8	25	20	1.2	310	Trace	.06	Trace	6
490. fresh, cucumber (as bread and butter pickles)	79.5	70	.9	.2	17.0	—	2.4	32	27	1.8	180	.02	.04	Trace	9
491. sour, cucumber or mixed	95.1	11	.5	.2	2.2	.4	2.0	25	20	1.2	310	Trace	.06	Trace	6
492. sweet, cucumber or mixed	70.5	108	.8	.4	26.4	—	1.9	16	18	1.3	110	(0)	.02	Trace	7
493 Pies, *apple	47.8	246	2.1	9.5	39.5	.7	1.1	7	24	.4	160	.03	.02	.2	1
494. *blueberry	52.7	216	2.1	6.9	37.5	.7	.8	10	22	.5	120	.02	.03	.2	4
495. *cherry	46.2	253	2.4	9.8	40.4	.2	1.2	10	27	.4	380	.03	.02	.2	1
496. coconut custard	58.5	204	5.2	8.7	26.3	.0	1.3	125	116	1.2	230	.05	.16	.3	(0)
497. custard	58.5	204	5.2	8.7	26.3	.0	1.3	125	116	1.2	230	.05	.16	.3	(0)
498. lemon meringue	47.4	252	3.6	10.1	37.4	.0	1.5	20	51	.5	170	.03	.08	.2	1
499. mince[37]	43.0	252	2.5	6.9	45.6	.5	2.0	16	40	2.2	10	.07	.04	.4	1
500. *pumpkin	58.9	202	4.2	9.6	25.8	.6	1.5	54	81	.8	1,910	.03	.12	.3	(0)
501. *Pie crust, plain, baked with unenriched flour	9.7	487	7.5	26.9	53.1	.2	2.8	11	65	.5	0	.03	.02	.5	(0)
502. enriched flour	9.7	487	7.5	26.9	53.1	.2	2.8	11	65	2.0	0	.22	.17	2.2	(0)
504. Pimientos, canned	92.4	27	.9	.5	5.8	.6	.4	7	17	1.5	2,300	.02	.06	.4	95
505. Pineapple, raw	85.3	52	.4	.2	13.7	.4	.4	16	11	.3	130	.08	.02	.2	24
506. canned, sirup pack, solids and liquid	78.0	78	.4	.1	21.1	.3	.4	29	7	.6	80	.07	.02	.2	9
508. Pineapple juice, canned	86.2	49	.3	.1	13.0	.1	.4	15	8	.5	80	.05	.02	.2	9
509. Plantain or baking banana, raw	66.4	119	1.1	.4	31.2	.4	.9	7	30	.7	[38]	.06	.04	.6	14
510. Plums (all, excluding prunes), raw	85.7	50	.7	.2	12.9	.5	.5	17	20	.5	350	.06	04	.5	5
511. Plums (Italian prunes), canned, sirup pack, solids and liquid (except pits)	78.6	76	.4	.1	20.4	.3	.5	8	12	1.1	230	.03	.03	.4	1
513. Popcorn, popped	4.0	386	12.7	5.0	76.7	2.2	1.6	(11)	(281)	(2.7)	(0)	(.39)	(.12)	(2.2)	(0)
515. Pork, fresh, packer's carcass, side, raw, medium	42.	457	11.9	45.	0.	0	.6	7	117	1.8	(0)	.58	.14	3.1	0
518. retail items[39], medium fat, ham, cooked	42.	400	24.	33.	0.	0	1.2	11	238	3.1	(0)	.53	.24	4.7	0
520. loin or chops, cooked	50.	333	23.	26.	0.	0	1.2	11	235	3.0	(0)	.83	.24	5.0	0
522. Pork, cured, ham, smoked[39], medium fat, raw	42.	389	16.9	35.	(.3)	0	5.4	10	136	2.5	(0)	.70	.19	4.0	0

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[36] Calcium may not be available because of presence of oxalic acid.

[37] The proximate constituents, calcium, phosphorus, and vitamin A are calculated from a recipe.

[38] The vitamin A values range from about 10 I.U. per 100 g. of white-fleshed plantains to 1,200 I.U. per 100 g. of deeper-yellow-fleshed varieties.

[39] Values for raw items are from the medium-fat wholesale cuts considered to be nearest approximations for indicated retail items.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
523. cooked	39.	397	23.	33.	(0.4)	0	5.4	10	166	2.9	(0)	0.54	0.21	4.2	0
524. luncheon meat, boiled ham	47.8	302	22.8	22.7	0.	0	6.7	9	92	2.7	(0)	1.01	.26	5.1	0
525. canned, spiced	55.2	289	14.9	24.3	1.5	.2	4.1	9	161	2.2	(0)	.32	.22	2.8	0
526. salt pork, fat, raw	8.	783	3.9	85.	0.	(0)	3.5	Trace	Trace	.6	(0)	(.18)	(.04)	(.9)	0
528. Potatoes, raw	77.8	83	2.0	.1	19.1	.4	1.0	11	56	.7	20	.11	.04	1.2	[40]17
529. cooked, baked	73.8	98	2.4	.1	22.5	.5	1.2	13	66	.8	20	.11	.05	1.4	17
530. boiled, unpeeled	77.8	83	2.0	.1	19.1	.4	1.0	11	56	.7	20	.10	.04	1.2	15
531. boiled, peeled before cooking	77.8	83	2.0	.1	19.1	.4	1.0	11	56	.7	20	.09	.03	1.0	14
532. *French fried	19.6	393	5.4	19.1	52.0	1.1	3.9	30	152	1.9	50	.18	.11	3.3	28
534. *hash-browned after holding overnight	50.7	241	3.3	11.7	31.9	.7	2.4	18	93	1.2	30	.08	.06	1.7	7
536. *mashed, milk and butter added	74.2	123	2.1	6.0	15.9	.3	1.8	27	59	.6	260	.08	.05	.8	7
537. steamed or pressure-cooked	77.8	83	2.0	.1	19.1	.4	1.0	11	56	.7	20	.10	.04	1.2	14
540. dehydrated	7.	357	7.1	.7	82.2	2.2	3.0	25	88	4.0	40	.30	.11	4.5	23
541. Potato chips	3.1	544	6.7	37.1	49.1	(1.1)	4.0	(30)	(152)	(1.9)	(50)	(.18)	(.11)	(3.2)	11
542. Potato flour	7.	357	7.1	.7	82.2	2.2	3.0	25	88	4.0	40	.30	.11	4.5	23
543. Pretzels	8.0	369	8.8	3.2	74.5	.3	5.5	(12)	(71)	(.7)	(0)	(.01)	(.04)	(.7)	(0)
544. Prunes, dried, unsulfured, uncooked	24.	268	2.3	.6	71.0	1.6	2.1	54	85	3.9	1,890	.10	.16	1.7	3
545. *cooked, no sugar added	64.6	125	1.1	.3	33.0	.8	1.0	25	40	1.8	890	.03	.08	.8	1
548. Prune juice, canned	80.	71	.4	0.	19.3	—	.3	(25)	(40)	(1.8)	—	(.03)	(.08)	.4	(1)
549. *Prune whip	58.7	148	2.8	.3	37.1	.7	1.1	26	42	1.8	860	.04	.11	.7	2
551. Pumpkin, canned	90.2	33	1.0	.3	7.9	1.2	.6	(20)	(36)	(.7)	3,400	.02	.06	.5	—
552. Radishes, raw	93.6	20	1.2	.1	4.2	.7	1.0	37	31	1.0	30	.03	.02	.3	24
553. Raisins, unsulfured, dried	24.	268	2.3	.5	71.2	—	2.0	78	129	3.3	50	.15	.08	.5	Trace
554. *cooked, sugar added, fruit and liquid	46.6	194	1.1	.2	51.1	—	1.0	38	63	1.6	20	.06	.04	.2	Trace
555. Raspberries, black, raw	80.6	74	1.5	1.6	15.7	6.8	.6	40	37	.9	0	.02	(.07)	(.3)	(24)
556. red, raw	84.1	57	1.2	.4	13.8	4.7	.5	40	37	.9	130	.02	(.07)	(.3)	24
559. Rhubarb, stems only, *cooked, sugar added	62.9	141	.4	.1	36.0	.6	.6	[41]41	20	.4	20	.01	—	.1	6
560. Rice, brown, raw	12.0	360	7.5	1.7	77.7	.6	1.1	39	303	2.0	(0)	.32	.05	4.6	(0)
561. converted, raw	12.3	362	7.6	.3	79.4	.2	.4	24	136	.8	(0)	.20	.03	3.8	(0)

562. *cooked	71.5	116	2.4	.1	25.4	.1	.6	8	43	.3	(0)	.05	.01	1.1	(0)
563. white or milled, raw	12.3	362	7.6	.3	79.4	.2	.4	24	136	.8	(0)	.07	.03	1.6	(0)
564. *cooked	70.5	119	2.5	.1	26.2	.1	.7	8	45	.3	(0)	.01	.01	.4	(0)
566. Rice products, flakes	3.5	392	5.9	.6	87.7	.5	2.3	21	116	1.8	(0)	.08	.08	.9	(0)
568. puffed	3.5	392	5.9	.6	87.7	.5	2.3	21	116	1.8	(0)	.08	.08	.9	(0)
569. puffed (added thiamine and niacin)	3.5	392	5.9	.6	87.7	.5	2.3	21	116	1.8	(0)	.46	.08	5.5	(0)
570. *Rolls, plain, unenriched (pan rolls)	28.5	309	9.0	5.5	55.1	.2	1.9	55	96	.7	0	.06	.11	1.0	(0)
571. enriched (pan rolls)	28.5	309	9.0	5.5	55.1	.2	1.9	55	96	[42]1.8	0	[42].24	[42].15	[42]2.2	(0)
572. sweet, unenriched	28.4	323	8.5	7.8	53.8	.2	1.5	63	104	.6	0	.05	.13	1.0	(0)
573. enriched	28.4	323	8.5	7.8	53.8	.2	1.5	63	104	[42]1.8	0	[42].24	[42].15	[42]2.2	(0)
575. Rutabagas, cooked	90.8	32	.8	.1	7.5	1.4	.8	55	41	.4	350	.05	.07	.7	21
576. Rye flour, light	11.	356	9.4	1.0	77.9	.4	.7	22	185	1.1	(0)	.15	.07	.6	(0)
577. medium	11.	326	11.4	1.7	74.8	1.0	1.1	(27)	262	2.6	(0)	.30	.12	2.5	(0)
578. dark	11.	318	16.3	2.6	68.1	2.4	2.0	54	(536)	4.5	(0)	.61	.22	2.7	(0)
579. Rye meal or whole grain	11.	321	12.1	1.7	73.4	2.0	1.8	(38)	376	3.7	(0)	.43	.22	1.6	(0)
580. Rye wafers or "Swedish health bread"	6.5	324	12.4	1.2	75.3	2.1	4.6	50	400	4.4	(0)	.32	.20	1.2	(0)
581. Salad dressings, commercial, plain (mayonnaise type)[43]	44.7	384	1.1	36.8	13.9	(0)	3.5	9	30	.4	140	.02	.03	(0)	0
582. French	39.6	394	.6	35.5	20.3	.3	4.0	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
584. mayonnaise[43]	16.	708	1.5	78.	3.0	(0)	1.5	19	60	1.0	210	.04	.04	(0)	0
586. Salmon, cooked, Pacific, broiled or baked	64.5	170	28.0	5.6	.2	0	1.7	—	(417)	(1.2)	—	.10	.28	8.1	—
587. canned, solids and liquid (incl. bones), Chinook or king	64.7	203	19.7	13.2	0.	0	2.4	[44]154	289	.9	230	.03	.14	7.3	(0)
588. chum	70.8	139	21.5	5.2	0.	0	2.6	[44]249	352	.7	60	.02	.16	7.1	(0)
589. coho or silver	67.6	166	21.1	8.4	0.	0	1.7	[44]232	254	.9	80	.03	.18	7.4	(0)
590. pink or humpback	70.0	143	20.5	6.2	0.	0	2.6	[44]187	286	.8	70	.03	.18	8.0	(0)
591. sockeye or red	67.2	173	20.2	9.6	0.	0	3.0	[44]259	344	1.2	230	.04	.16	7.3	(0)
592. Sardines, Atlantic type, canned in oil, solids and liquid	47.1	338	21.1	27.0	1.0	—	3.9	354	434	3.5	—	(.01)	(.14)	(3.9)	(0)
593. drained solids	57.4	214	25.7	11.0	1.2	—	(4.7)	386	586	2.7	220	.02	.17	4.8	(0)
594. pilchards, Pacific type, canned, solids and liquid, natural pack	65.2	200	17.7	13.5	.7	0	2.9	(381)	(168)	4.1	(30)	(.01)	(.30)	(7.4)	(0)

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[40] Year round average. Recently dug potatoes contain about 24 mg. ascorbic acid per 100 g. The value is only half as high after three months of storage and about one-third as high when potatoes have been stored as long as six months.

[41] Calcium may not be available because of presence of oxalic acid.

[42] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in the standards of identity of breads proposed by the Federal Security Agency and published in the Federal Register, August 3, 1943.

[43] Minerals and vitamins are calculated from a recipe.

[44] If bones are discarded, calcium content would be much lower. Bones equal about 2 per cent of total contents of can.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
595. tomato sauce	63.1	216	17.8	14.8	1.7	0.2	2.7	381	168	4.1	30	0.01	0.27	5.3	(0)
596. Sauerkraut, canned, solids and liquid	93.2	16	1.1	.2	3.4	.7	2.1	36	18	(.5)	30	.03	.06	.1	16
597. drained solids	91.2	22	1.4	.3	4.4	.9	2.7	36	18	(.5)	40	.03	.06	.1	16
598. Sausage, Bologna	62.4	221	14.8	15.9	3.6	—	3.3	(9)	(112)	2.2	(0)	.18	.19	2.7	0
599. Frankfurter, raw	60.0	257	14.2	20.5	2.7	—	2.7	8	100	1.5	(0)	.18	.19	2.8	0
600. cooked	62.	248	14.	20.	2.	—	2.	6	49	1.2	(0)	.16	.18	2.5	0
601. liver, liverwurst	59.0	263	16.7	20.6	1.5	—	2.2	9	238	5.4	5,750	.17	1.12	4.6	(0)
602. pork, links or bulk, raw	41.9	450	10.8	44.8	0.	0	2.1	6	100	1.6	(0)	.43	.17	2.3	0
603. pork, bulk, canned	55.4	299	15.4	25.9	0.	0	3.3	9	166	2.3	(0)	.20	.24	3.0	0
604. Vienna sausage, canned	64.2	215	15.8	16.4	0.	0	3.6	9	170	2.4	(0)	.10	.12	3.1	0
605. Scallops, raw (edible muscle)	80.3	78	14.8	.1	3.4	0	1.4	26	208	1.8	0	(.04)	.10	1.4	—
606. Shad or American shad, raw	70.2	168	18.7	9.8	0.	0	1.4	—	260	.5	—	(.15)	.24	(8.4)	—
607. *Sherbet	68.1	123	1.5	.0	30.0	—	.4	50	40	.0	0	.02	.08	Trace	(0)
608. *Shortbread	4.0	504	6.8	24.4	64.3	.2	.5	10	59	.4	0	.04	.02	.5	(0)
609. Shrimp, canned, dry pack or drained solids of wet pack	66.2	127	26.8	1.4	—	—	5.8	115	263	3.1	60	.01	.03	2.2	(0)
610. wet pack, solids and liquid	75.6	89	18.7	.9	.3	—	4.5	59	152	1.8	60	.01	.03	1.4	(0)
611. Sirup, table blends (chiefly corn sirup)	25.	286	(0)	(0)	(74.)	—	.6	46	16	4.1	0	0	.01	.1	(0)
613. Soups, canned ready-to-serve,[45] bean	82.4	76	3.4	2.0	11.8	.6	.4	38	41	1.1	—	.04	.04	.3	—
615. beef	91.6	40	2.4	1.4	4.4	.1	.2	6	25	.2	—	—	—	—	—
617. bouillon, broth, and consomme	95.	4	(1.)	—	(0.)	0	1.5	1	10	.4	0	0	.02	.3	0
619. chicken	93.6	30	1.4	1.0	3.8	.1	.2	8	8	.2	—	.01	.05	.6	—
622. clam chowder	90.8	34	1.8	.9	4.9	.2	1.6	14	30	1.4	—	—	—	—	—
624. cream soup (asparagus, celery, or mushroom)	84.8	79	2.8	4.6	7.2	.1	.6	85	69	.2	80	.02	.08	Trace	(0)
627. noodle, rice, or barley	90.4	47	2.4	1.8	5.2	.2	.2	33	34	.1	10	.01	.02	.3	0
629. pea	86.2	57	2.6	.8	10.2	.6	.2	13	40	.6	(180)	.07	.03	.5	2
631. tomato	90.7	37	.9	.9	7.3	.2	.2	10	16	.4	(500)	.01	.04	.3	4
633. vegetable	91.6	33	1.7	.7	5.8	.3	.2	13	20	.3	—	.02	.03	.4	3
636. Soups, dehydrated, navy bean[46]	7	327	17.6	1.2	62.9	3.5	11.3	134	425	9.2	0	.42	.19	2.2	1

637. pea[47]	7.	328	20.4	1.1	61.0	1.1	10.5	67	364	5.4	220	.57	.19	3.1	1
638. Soybeans, whole, mature, dried	7.5	331	34.9	18.1	[48]34.8	5.0	4.7	227	586	8.0	110	1.07	.31	2.3	Trace
639. Soybean curd	85.1	71	7.0	4.1	3.0	.1	.8	100	95	1.5	—	.06	.05	.4	(0)
640. Soybean flour, flakes, grits, low fat	11.	228	44.7	1.1	[48]37.7	2.3	5.5	265	623	13.0	70	1.10	.35	2.9	(0)
641. medium fat	9.	264	42.5	6.5	[48]37.2	2.6	4.8	244	610	13.0	110	.82	.34	2.6	(0)
642. full fat	9.	347	35.9	20.6	[48]29.9	2.3	4.6	195	553	12.1	140	.77	.28	2.2	(0)
643. Soybean milk (without added calcium and vitamins)	92.5	33	3.4	1.5	2.1	.0	.5	21	47	.7	—	.09	.04	.3	(0)
644. Soybean sprouts, raw	86.3	46	6.2	1.4	5.3	.8	.8	48	67	1.0	180	.23	.20	.8	13
646. Spaghetti, *cooked	60.6	149	5.1	.6	30.2	.2	3.5	9	65	.6	(0)	.02	.02	.5	(0)
647. enriched, dry	8.6	377	12.8	1.4	76.5	.4	.7	22	165	[49]2.9	(0)	[49].88	[49].37	[49]6.0	(0)
648. *cooked	60.6	149	5.1	.6	30.2	2	3.5	9	65	1.1	(0)	.17	.10	1.4	(0)
649. Spinach, raw	92.7	20	2.3	.3	3.2	.6	1.5	[50]81	55	3.0	9,420	.11	.20	.6	59
650. cooked	90.8	26	3.1	.6	3.6	1.0	1.9	[50]124	33	2.0	11,780	.08	.20	.6	30
656. Squash, summer, cooked, diced	95.0	16	.6	.1	3.9	.5	.4	15	15	.4	260	.04	.07	.6	11
659. Squash, winter, cooked, baked, mashed	85.7	47	1.9	.4	11.0	1.8	1.0	24	35	.8	6,190	.05	.15	.6	7
660. cooked, boiled, mashed	88.6	38	1.5	.3	8.8	1.4	.8	19	28	.6	4,950	.04	.10	.4	5
662. Starch, pure (including arrowroot, corn, etc.)	12.	362	.5	.2	87.	.1	.3	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
663. Strawberries, raw	89.9	37	.8	.5	8.3	1.4	.5	28	27	.8	60	.03	.07	.3	60
665. Sugars, granulated, cane or beet	.5	385	(0.)	(0.)	99.5	(0)	—	—	—	—	(0)	(0)	(0)	(0)	(0)
666. powdered	.5	385	(0.)	(0.)	99.5	(0)	—	—	—	—	(0)	(0)	(0)	(0)	(0)
667. brown	3.	370	(0.)	(0.)	95.5	—	1.2	[51]76	[51]37	2.6	(0)	(0)	(0)	(0)	(0)
668. corn sugar unrefined	7.5	348	—	—	(90.)	—	.3	—	—	—	(0)	(0)	(0)	(0)	(0)
669. dextrose (including refined corn sugar), anhydrous	.5	385	(0.)	(0.)	99.5	(0)	—	—	—	—	(0)	(0)	(0)	(0)	(0)
670. crystallized	10.	348	(0.)	(0.)	90.	(0)	—	—	—	—	(0)	(0)	(0)	(0)	(0)
671. maple	7.5	348	—	—	(90.)	—	.9	—	—	—	—	—	—	—	—
673. Sweet potatoes,[52] cooked, baked	61.1	152	2.2	.9	34.4	1.2	1.4	37	60	.9	9,510	.10	.06	.8	23

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[45] All the ready-to-serve soups are calculated from equal weights of the condensed soup and water except cream soup which was based on equal weights of the condenses soup and milk.

[46] Navy bean meal with farinaceous flour up to 15 per cent.

[47] Pea meal with farinaceous flour up to 15 per cent.

[48] Approximately 40 per cent of this total amount of carbohydrate calculated by difference is sugar, starch, and dextrin. The remaining portion is made up of materials thought to be utilized only poorly, if at all, by the body.

[49] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in the standards of identity promulgated under the Food, Drug, and Cosmetic Act.

[50] Calcium may not be available because of presence of oxalic acid.

[51] Calcium and phosphorus are based on dark brown sugar; values would be lower for light brown sugar.

[52] If very pale varieties only were used, the vitamin A value would be very much lower.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
674. boiled	68.5	123	1.8	0.7	27.9	1.0	1.1	30	49	0.7	7,700	0.09	0.05	0.6	20
675. candied	57.4	179	1.5	3.6	36.2	.8	1.3	36	45	.9	6,250	.04	.04	.5	9
679. Swordfish, cooked, broiled	64.8	178	27.4	6.8	0.	0	1.7	20	251	1.1	2,300	.05	.06	10.3	(0)
680. Tangerines (including other Mandarin type oranges)	87.3	44	.8	.3	10.9	1.0	.7	(33)	(23)	(.4)	(420)	.07	(.03)	(.2)	31
681. Tangerine juice, unsweetened, fresh	89.2	39	.9	.3	9.2	—	.4	19	16	(.2)	(420)	.07	(.03)	.2	31
683. Tapioca, dry	12.6	360	.6	.2	86.4	.1	.2	12	12	(1.0)	(0)	(0)	(0)	(0)	(0)
684. Tomatoes, raw	94.1	20	1.0	.3	4.0	.6	.6	11	27	.6	1,100	.06	.04	.5	23
685. canned or cooked	94.2	19	1.0	.2	3.9	.4	.7	(11)	(27)	(.6)	1,050	.06	.03	.7	16
686. Tomato juice, canned	93.5	21	1.0	.2	4.3	.2	1.0	(7)	(15)	(.4)	1,050	.05	.03	.8	16
687. Tomato catsup	69.5	98	2.0	.4	24.5	.4	3.6	12	18	.8	(1,880)	.09	.07	2.2	11
688. Tomato flakes	3.	340	10.8	3.3	76.7	6.5	6.2	119	293	6.5	3,720	.65	.43	6.5	114
689. Tomato puree, canned	89.2	36	1.8	.5	7.2	.4	1.3	(11)	(37)	(1.1)	1,880	.09	.07	1.8	28
690. Tongue, beef, medium fat, raw	68.	207	16.4	15.	.4	(0)	.9	9	187	2.8	(0)	.12	.29	5.0	(0)
691. Tortillas	41.9	211	5.8	(2.8)	48.6	(1.4)	.9	111	184	2.2	[53]210	.19	.06	1.0	—
692. Tuna fish, canned, solids and liquid	52.5	290	23.8	20.9	0.	0	2.3	7	294	1.2	(220)	(.04)	(.10)	(10.8)	(0)
693. drained solids	60.0	198	29.0	8.2	0.	0	2.7	(8)	(351)	1.4	80	.05	.12	12.8	(0)
694. Turkey, medium fat, raw	58.3	268	20.1	20.2	0.	0	1.0	23	320	3.8	Trace	.09	.14	8.0	(0)
696. Turnips, cooked	92.3	27	.8	.2	6.0	1.2	.7	40	34	.5	Trace	.04	.06	.4	18
698. Turnip greens, cooked in small amount of water until tender	89.5	30	2.9	.4	5.4	1.2	1.8	259	50	2.4	10,600	.06	.41	.7	60
702. Veal, carcass or side excluding kidney fat, raw, medium fat	68.	190	19.1	12.	0.	0	1.0	11	193	2.9	(0)	.14	.25	6.4	0
705. retail items[54] medium fat: cutlet, boned (wholesale round), cooked	60.	219	28.	11.	0.	0	1.4	12	258	3.5	(0)	[55] .08	[55] .28	[55]6.1	0
707. shoulder roast, boned (wholesale chuck), cooked	59.	228	28.	12.	0.	0	1.4	12	258	3.6	(0)	.13	.31	7.9	0
709. stew meat, without bone, cooked	53.	296	25	21.	0.	0	.8	11	124	3.0	(0)	[56] .05	[56] .24	[56]4.6	0
712. Vinegar	—	12	0	—	(5.0)	(0)	.3	7	10	.5	—	—	—	—	—

713. *Waffles, baked with unenriched flour	40.	287	9.3	10.6	37.8	0.1	2.3	192	204	1.0	360	0.06	0.18	0.4	(0)
714. with enriched flour	40.	287	9.3	10.6	37.8	.1	2.3	192	204	1.8	360	.18	.27	1.3	(0)
715. Walnuts, Persian or English	3.3	654	15.0	64.4	15.6	2.1	1.7	83	380	2.1	30	.48	.13	1.2	3
716. Watermelons	92.1	28	.5	.2	6.9	.6	.3	7	12	.2	590	.05	.05	.2	6
717. Wheat, whole grain,[57] hard red spring	13.0	330	14.0	2.2	69.1	2.3	1.7	36	383	3.1	(0)	.57	.12	4.3	(0)
718. hard red winter	12.5	330	12.3	1.8	71.7	2.3	1.7	46	354	3.4	(0)	.52	.12	4.3	(0)
719. soft red winter	14.0	326	10.2	2.0	72.1	2.3	1.7	42	400	3.5	(0)	.43	.11	(3.6)	(0)
720. white	11.5	335	9.4	2.0	75.4	1.9	1.7	36	394	3.0	(0)	.53	.12	5.3	(0)
721. durum	13.0	332	12.7	2.5	70.1	1.8	1.7	37	386	4.3	(0)	.66	.12	(4.4)	(0)
722. Wheat flours, whole (from hard wheats)	12.	333	13.3	2.0	71.0	2.3	1.7	41	372	3.3	(0)	.55	.12	4.3	(0)
723. 80 per cent extraction (from hard wheats)	12.	365	12.	1.3	74.1	.5	.65	24	191	1.3	(0)	.26	.07	2.0	(0)
724. straight, hard wheat	12.	365	11.8	1.2	74.5	.4	.46	20	97	1.4	(0)	.12	.07	1.4	(0)
725. straight, soft wheat	12.	364	9.7	1.0	76.9	.4	.42	20	97	1.1	(0)	.08	.05	1.2	(0)
726. self-rising, unenriched	12.	350	9.2	1.0	73.8	.4	4.0	272	484	1.0	(0)	.08	.05	1.1	(0)
727. enriched	12.	350	9.2	1.0	73.8	.4	4.0	[58]272	484	[58]2.9	(0)	[58].44	[58].26	[58]3.5	(0)
728. patent, all-purpose or family flour, unenriched	12.	364	10.5	1.0	76.1	.3	.43	16	87	.8	(0)	.06	.05	.9	(0)
729. enriched	12.	364	10.5	1.0	76.1	.3	.43	16	87	[59]2.9	(0)	[59].44	[59].26	[59]3.5	(0)
730. bread flour, unenriched	12.	365	11.8	1.1	74.7	.3	.44	16	95	.9	(0)	.08	.06	1.0	(0)
731. enriched	12.	365	11.8	1.1	74.7	.3	.44	16	95	[59]2.9	(0)	[59].44	[59].26	[59]3.5	(0)
732. cake or pastry flour	12.	364	7.5	.8	79.4	.2	.31	17	73	.5	(0)	.03	.03	.7	(0)
733. Wheat products, flakes	3.8	355	10.8	1.6	80.2	1.7	3.6	46	329	3.0	(0)	.08	.18	4.8	(0)
734. flakes (added iron, thiamine, and niacin)	3.8	355	10.8	1.6	80.2	1.7	3.6	46	329	4.2	(0)	.56	.18	6.4	(0)
735. germ	11.0	361	25.2	10.0	49.5	2.5	4.3	84	1,096	8.1	(0)	2.05	.80	4.6	(0)
736. puffed	3.8	355	10.8	1.6	80.2	1.7	3.6	46	329	3.0	(0)	.08	.18	4.8	(0)
737. puffed (added iron, thiamine, and niacin)	3.8	355	10.8	1.6	80.2	1.7	3.6	46	329	4.2	(0)	.56	.18	6.4	(0)
739. rolled, *cooked	79.7	75	2.2	.4	16.9	.5	.8	8	76	.7	(0)	.07	.03	.9	(0)
740. shredded, plain	5.6	360	10.1	2.5	80.1	2.3	1.7	47	360	3.5	(0)	.22	.12	4.4	(0)
742. whole meal, dry	8.2	344	12.7	1.7	75.3	2.2	2.1	46	392	3.4	(0)	.55	.15	4.4	(0)
743. *cooked	80.3	72	2.7	.3	15.8	.4	.9	9	83	.7	(0)	.10	.03	.9	(0)

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[53] Vitamin A value of tortillas made from yellow corn; tortillas made from white corn have no vitamin A value.

[54] Values for raw items are from the medium fat wholesale cuts considered to be nearest approximations for indicated retail items.

[55] Data assume cut to be prepared by braising or pot-roasting. Use of proportionate quantity of drippings would add approximately 50 per cent more thiamine and niacin and 25 per cent more riboflavin.

[56] Use of proportionate quantity of liquid would double amount of thiamine and niacin and add one-third more riboflavin.

[57] Figures for moisture are based on product as it reaches the mill prior to tempering; other proximate constituents are adjusted to this basis.

[58] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in the standards of identity promulgated under the Food, Drug, and Cosmetic Act. Calcium is based on the level usually found in self-rising flour which is in excess of the minimum (500 mg. per pound) required.

[59] Iron, thiamine, riboflavin, and niacin are based on the minimum level of enrichment specified in the standards of identity promulgated under the Food, Drug, and Cosmetic Act.

COMPOSITION OF FOODS, 100 G., EDIBLE PORTION—(*Continued*)

Food and description	*Water*	*Food energy*	*Protein*	*Fat*	*Carbohydrate*		*Ash*	*Calcium*	*Phosphorus*	*Iron*	*Vitamin A value*	*Thiamine*	*Riboflavin*	*Niacin*	*Ascorbic acid*
					Total	*Fiber*									
	Pct.	*Cal.*	*g.*	*g.*	*g.*	*g.*	*g.*	*mg.*	*mg.*	*mg.*	*I.U.*	*mg.*	*mg.*	*mg.*	*mg.*
744. **whole meal (added wheat germ, iron, and thiamine), dry**	11.0	336	12.8	2.0	72.4	1.8	1.8	50	400	30.0	(0)	1.50	.16	5.2	(0)
745. *cooked	85.1	55	2.1	.3	11.8	.3	.7	8	65	4.9	(0)	.22	.03	.8	(0)
746. Whey, fluid	93.2	26	.9	.3	5.1	0	.5	51	53	.1	10	.04	.15	.1	—
747. dried	6.2	344	12.5	1.2	72.4	0	7.7	679	576	—	50	.49	2.50	.8	—
748. *White sauce, medium	73.5	162	4.0	12.5	8.8	.0	1.4	115	95	.1	510	.03	.15	.1	Trace
749. Wild rice, parched, raw	8.5	364	14.1	.7	75.3	1.0	1.4	19	339	—	(0)	.45	.63	6.2	(0)
750. Yeast, compressed, baker's	70.9	86	(10.6)	.4	13.0	.3	2.4	25	605	4.9	(0)	.45	2.07	28.2	(0)
751. dried, brewer's	7.0	273	(36.9)	1.6	37.4	.8	7.9	106	1,893	18.2	(0)	9.69	5.45	36.2	(0)

IV. AVERAGE PORTIONS OF FOODS

Caloric Values and Acid- and Base-Forming Effects

The data presented here are reproduced by permission from the 1946 Nutritional Charts prepared by the H. J. Heinz Co., Pittsburgh, Pa. The alkaline or acid effect is given in terms of ml. of N alkali (+) or acid (−) corresponding to 100 g. or 100 ml. of food (see discussion on p. 1097). The measure of "average portion" as given is based upon the edible portion only and upon articles of average size. The food is calculated as in the raw state. These figures are not offered as exact data but merely as a guide in estimating average portions or servings; naturally there is considerable possible variation in this respect. For precise nutritional studies, all food consumed should be accurately measured. In these tables, T. = tablespoon, t. = teaspoon, c. = cup.

VEGETABLES

Name	*Alkaline (+) or Acid (−) Effect*	*Average Portion*		
		Total Calories	*Measure*	*Weight*
				grams
Artichokes (globe)	+ 7.6	50	1 heart, edible leaf portion	100
Asparagus	+ 0.8	23	6 stalks	100
Bamboo shoots	+ 8.0	30	¾ c.	100
Beans, baked	+ 6.0	99	½ c.	100
Beans, dried	+18.0	94	2 T. shelled	28
Beans, green	+ 5.4	37	1 c. stringless	100
Beans, green kidney	+18.0	88	⅔ c. shelled	100
Beans, dried lima	+42.0	91	⅛ c. shelled	28
Beans, green lima	+28.0	125	⅔ c. shelled	100
Beans, soy, dried	+ 4.5	119	3 T. shelled	28
Beans, soy, green	+17.0	158	⅔ c. shelled	100
Beans, soy, sprouts	+16.0	66	1 c.	100
Beets	+11.0	42	⅔ c.	100
Beet greens	+27.0	28	1 c.	100
Broccoli	+ 9.3	32	1 c. "curd"	100
Brussels sprouts	+11.0	53	6	100
Cabbage	+ 6.0	14	¾ c. shredded as slaw	57
Carrots	+11.0	40	1 large, scraped	100
Cauliflower	+ 5.3	27	1 c. "curd"	100
Celeriac (celery root)	+ 8.8	39	¾ c. pared	100
Celery	+ 7.8	8	2 stalks	40
Chard, leaves	+16.0	22	1½ c.	100
Chives	+13.0	52	2 bunches	100
Collards	+	45	⅔ c.	100
Corn, canned (yellow)	− 1.8	84	½ c.	115
Corn, green (yellow)	− 2.0	104	½ c. cut from cob	100
Cucumbers	+ 7.9	7	10 slices, pared	57
Dandelion greens	+18.0	45	1 c.	100
Eggplant	+ 6.3	25	2 slices, pared	100
Endive	+ 7.0	9	½ head	45
Escarole (chicory)	+ 4.0	3	¼ head	16
Garlic	. .	. .	1 clove, peeled	2
Horseradish	+ 4.8	9	1 t.	10

VEGETABLES—(*Continued*)

Name	*Alkaline (+) or Acid (−) Effect*	*Average Portion*		
		Total Calories	*Measure*	*Weight*
				grams
Kale	+ 7.7	45	1 c. leaves	100
Kohlrabi	+ 8.0	32	½ c.	100
Lambsquarters	..	44	1 c. leaves	100
Leeks	+ 7.0	23	2 stalks	57
Lentils, dried	−16.0	94	2 T.	28
Lettuce	+ 7.4	12	¼ head	75
Marrow, vegetable	+ 1.9	17	⅝ c.	100
Mushrooms	+ 4.0	30	7	100
Mustard greens	..	25	1 c. leaves	100
Okra	+ 4.5	17	5 pods	50
Onions	+ 1.5	23	1	50
Parsley	..	..	1 sprig	1
Parsnips	+12.0	75	½ large, scraped	100
Peas, dried	+ 5.0	92	2 T.	28
Peas, green	+ 1.3	92	¾ c. shelled	100
Peppers, green	+ 1.7	24	1 empty pod	100
Potatoes, sweet	+ 6.7	175	1 pared	145
Potatoes, white	+ 7.0	101	1 pared	120
Pumpkins	+ 1.5	31	½ c. seeded, rind removed	100
Radishes	+ 2.9	7	5	35
Rhubarb	+ 8.5	15	1 c. stems	100
Rutabagas	+ 8.5	36	¾ c. scraped	100
Salsify (oyster plant)	+ 2.9	78	2 scraped	100
Sauerkraut	+ 5.7	14	⅔ c.	100
Spinach	+27.0	22	1 c. leaves	100
Squash, summer	+ 1.0	17	1 c. seeded, rind removed	100
Squash, winter	+ 2.6	38	1 c. seeded, rind removed	100
Taro	+18.0	93	1 corm, pared	100
Tomatoes	+ 5.6	20	1 small, cored	100
Turnips	+ 2.7	30	¾ c. pared	100
Turnip greens	+ 2.3	32	1 c. leaves	100
Water cress	+12.0	21	2½ c. leaves	100
Yams	+	155	1 tuber, pared	150
Yautia, yellow	+15.0	110	1 corm, pared	100

FRUITS

Name	Alkaline (+) or Acid (–) Effect	Average Portion		
		Total Calories	Measure	Weight
				grams
Apples	+ 3.7	90	1 large, cored	150
Apricots	+ 6.1	54	4 halves, stoned	100
Avocados	+11.0	259	½ pear, pared, stoned	100
Bananas	+ 5.6	96	1 small, peeled	100
Blackberries	+ 6.9	46	1 c.	100
Blueberries (huckleberries)	+ 2.7	63	⅔ c.	100
Cherries	+ 4.5	67	18 stoned	100
Cranberries	– †	48	1 c.	100
Currants	+ 6.3	48	1 c.	100
Dates, dried	+11.0	92	4 stoned	30
Figs, dried	+33.0	83	1½	30
Gooseberries	+ 3.3	37	⅔ c.	100
Grapefruit	+ 7.0	43	½ c. juice	100
Grapes	+ 4.0	72	1 bunch, seeded	100
Guavas	+ 8.0	56	1 pared, seeded	100
Lemons	+ 5.0	40	½ c. juice	100
Limes	+10.0	52	½ c. juice	100
Loganberries	+ 7.3	64	1 c.	100
Mangoes	+ 5.0	69	½ pared, seeded	100
Melon—Cantaloupes	+ 7.5	40	½ seeded, rind removed	200
Honeydew	. .	68	⅙ seeded, rind removed	200
Muskmelons	+ 7.5	52	½ seeded, rind removed	200
Watermelons	+ 2.7	58	1 slice, seeded	200
Nectarines	+ 6.2	65	2 pared, stoned	100
Olives, green	+ ‡	35	5 small, stoned	25
Oranges	+ 5.6	48	½ c. juice	100
Papayas	. .	40	½ seeded, rind removed	100
Peaches (yellow)	+ 5.9	49	1 large, pared, stoned	100
Pears	+ 4.2	64	2 halves, cored, pared	100
Persimmons	. .	135	1 small, seeded	100
Pineapples	+ 6.8	57	2 slices, canned	100
Plums	– †	54	3 stoned	100
Pomegranates	+ 3.5	74	½ seeded	100
Prunes, dried	– †	108	4 stewed, stoned	37
Quinces	+ 4.9	51	1 boiled, as a sauce	100
Raisins	+34.0	131	⅓ c. seeded and seedless	45
Raspberries, black	+ 3.8	69	⅞ c.	100
Raspberries, red	+ 6.0	56	⅞ c.	100
Strawberries	+ 5.5	36	12 hulled	100
Tangerines	+ 5.3	46	2 peeled, seeded	100

CEREALS AND BAKERY PRODUCTS

Name	Alkaline (+) or Acid (−) Effect	Total Calories	Average Portion	
			Measure	Weight
				grams
Barley, pearled	−10.0	107	3 T.	30
Bread, enriched white	− 4.0	130	2 slices	50
Bread, rye	− 5.2	125	3 slices	50
Bread, white	− 4.0	130	2 slices	50
Bread, whole wheat	− 3.6	129	2 slices	50
Cake, devils food, iced	− 6.3	202	1 piece	57
Cake, sponge	− 9.7	108	1 piece	37
Cookies (average)	− or +	106	1–2	28
Cornmeal, yellow	− 4.9	107	3 T.	30
Crackers (Graham)	− 8.5	125	3	30
Crackers, soda	− 9.0	104	4	25
Doughnuts	− 1.7	242	1	57
Farina	−11.0	110	3 T.	30
Farina, enriched	−11.0	110	3 T.	30
Flour, buckwheat	− 7.1	77	2 T.	22
Flour, rye	−11.0	64	2 T.	18
Flour, soybean	+ 9.5	82	2 T.	18
Flour, white	− 9.0	60	2 T.	17
Flour, whole wheat	−11.0	57	2 T.	16
Hominy, white	− 1.7	178	¼ c.	50
Macaroni or spaghetti	−14.0	101	¼ c.	28
Noodles, egg	−	382	¾ c.	100
Oatmeal (Rolled Oats)	−12.0	98	¼ c.	25
Popcorn, popped	− 8.0	69	1 c.	17
Pretzels	− 7.0	90	6	25
Rice, brown	− 5.7	106	3 T.	30
Rice, white	− 9.0	98	2 T.	28
Tapioca	0.0	140	¼ c.	40
Wheat bran	−25.0	87	1 c.	28
Wheat germ	−20.0	76	2 T.	20
Wheat, whole	−12.0	72	2 T.	20

MEATS, FISH, AND POULTRY PRODUCTS

				grams
Bacon (lean)	− 4.8	149	4 slices	28
Beef brains	−21.0	144	¼ lb.	113
Beef, chuck	−11.0	303	¼ lb.	113
Beef, corned (medium)	−10.0	325	¼ lb.	113
Beef heart (lean)	− 9.1	118	¼ lb.	113

MEATS, FISH, AND POULTRY PRODUCTS—(*Continued*)

Name	*Alkaline (+) or Acid (−) Effect*	*Average Portion*		
		Total Calories	*Measure*	*Weight*
				grams
Beef kidney	− 8.5	155	1 c.	113
Beef liver	−11.0	149	¼ lb.	113
Beef, loin	−11.0	385	¼ lb.	113
Beef steak	−11.0	263	¼ lb.	113
Beef sweetbreads	−12.0	463	1 c.	113
Beef tongue	−11.0	170	5 slices	75
Bluefish	− 9.0	133	1 fish filet	113
Bologna	− 8.5	93	4 slices, skin removed	43
Chicken	−14.0	141	¼ lb.	113
Clams	..	87	9	113
Codfish	− 8.4	79	¼ lb. filet	113
Crabs	−40.0	92	⅔ c.	113
Duck	−24.0	180	¼ lb.	113
Egg white	− 5.2	16	1 white	35
Egg yolk	−27.0	60	1 yolk	17
Eggs	−11.0	82	1, shell removed	52
Frankfurters	− 9.3	227	2 links	113
Gelatin, dried	−	31	1 T.	9
Goose	− 7.8	173	¼ lb.	113
Haddock	−12.0	81	¼ lb. filet	113
Halibut	− 9.3	137	¼ lb. filet	113
Ham (fat)	− 9.5	515	¼ lb.	113
Herring	− 9.0	154	1 filet	113
Lamb chops	− 9.7	260	2 chops	113
Lamb, leg	− 9.6	260	¼ lb.	113
Lobster	−38.0	95	⅔ c. canned	113
Mackerel	−11.0	207	1 filet	113
Margarine, fortified	0.0	103	1 T.	14
Mince meat	+12.0	316	¼ lb.	113
Mutton, leg	− 9.6	216	¼ lb.	113
Oysters	−23.0	92	6	113
Pork chops	− 8.0	392	2 chops	113
Pork sausage	− 7.5	156	2 links	35
Rabbit	−15.0	198	¼ lb.	113
Salmon	−11.0	246	1 c. canned	113

MEATS, FISH, AND POULTRY PRODUCTS—(*Continued*)

Name	*Alkaline (+) or Acid (−) Effect*	*Average Portion*		
		Total Calories	*Measure*	*Weight*
				grams
Sardines in oil	−11.0	104	4 sardines	50
Scallops	−36.0	84	⅔ c.	113
Shrimp	− 1.6	72	8	65
Tripe	− 8.1	106	¼ lb.	113
Tuna in oil	−	111	½ c., canned	57
Turkey	−11.0	176	¼ lb.	113
Veal chops	−14.0	236	2 chops	113
Veal cutlet	− 9.8	208	¼ lb.	113
Whitefish	−10.0	170	¼ lb. filet	113

DAIRY PRODUCTS

				grams
Butter	0.0	103	2 pats	14
Buttermilk	+ 2.2	74	1 glass	210
Cheese, American	− 5.4	110	1 slice	28
Cheese, Cheddar	− 5.0	110	1 cube	28
Cheese, Cottage	− 4.5	28	⅛ c.	28
Cheese, Cream	− 3.4	104	1 cube	28
Cheese, Roquefort	+	109	1 slice	28
Cheese, Swiss	− 5.0	113	1 slice	28
Cream, sour	−	29	1 T.	15
Cream, sweet	0.0	31	1 T.	15
Ice cream, vanilla	+ 0.2	214	½ c.	100
Milk, chocolate	+ 1.5	174	1 glass	210
Milk, condensed (sweetened)	+ 5.2	98	2 T.	30
Milk, dried skim	+18.0	57	2 T.	16
Milk, dried whole	+12.0	80	2 T.	16
Milk, evaporated	+ 5.1	139	½ glass	115
Milk, goat	+	147	1 glass	210
Milk, human	+	68		100
Milk, skim	+ 2.4	76	1 glass	210
Milk, whole (pasteurized)	+ 2.3	145	1 glass	210
Whey, dried	+	28	1 T.	8

NUTS

				grams
Almonds	+12.0	88	12 nuts	14
Brazil nuts	+ 4.5	96	2 nuts	14
Butternuts	. .	95	4 nuts	14
Cashew	. .	85	10 nuts	14
Chestnuts, fresh	+ 5.0	26	3 nuts, skin removed	14

NUTS—(*Continued*)

Name	*Alkaline (+) or Acid (−) Effect*	*Average Portion*		
		Total Calories	*Measure*	*Weight*
				grams
Coconut, dried	+ 8.3	79	2 T.	14
Hazelnuts (filberts)	+ 4.8	92	10 nuts	14
Hickory	. .	99	12 nuts	14
Peanuts	− 3.9	83	16 nuts, skin removed	14
Peanut butter	− 3.9	104	1 T.	17
Pecans	− 5.6	103	12 meats	14
Pistachios	. .	87	⅛ c.	14
Walnuts, black	. .	93	12 meats	14
Walnuts, English	− 7.8	97	12 meats	14

MISCELLANEOUS FOODS

Name	*Alkaline (+) or Acid (−) Effect*	*Total Calories*	*Measure*	*Weight*
				grams
Apple Pie	+ 3.0	346	1 piece, ⅙ pie	160
Chocolate, sweetened	+ 2.4	313		57
Chocolate, unsweetened	+ 6.7	32	1 T.	5
Cocoa	+11.0	27	2 t.	6
Codliver oil	. .	99	1 T.	11
Coffee*	+63.0	. .	1 c. infusion	180
Corn oil	0.0	99	1 T.	11
Corn syrup	0.0	65	1 T.	22
Cottonseed oil	0.0	99	1 T.	11
Honey	− 1.1	80	1 T.	25
Ketchup, tomato	+	22	1 T.	20
Lard	0.0	126	1 T.	14
Maple syrup	+	56	1 T.	22
Marmalade, orange	+ 2.8	85	1 T.	25
Molasses	+60.0	40	2 t.	14
Olive oil	0.0	99	1 T.	11
Pickles	−	24	4 small	28
Potato chips	+21.0	97	8–10 large pieces	17
Succotash	+	94	⅓ c.	100
Sugar				
Granulated (sucrose)	0.0	32	2 t.	8
Brown	+60.0	19	2 t.	5
Tea*	+47.0	. .	1 c. infusion	180
Yeast, fresh	. .	15	1 cake	14
Yeast, dried brewers'	+17.1	35	1 T.	10

---

* The infusion has little food value except for added sugar and cream. The analytical figures apply to dried coffee bean and dried tea leaves.

† These foods are alkaline, but because of substances in them which give rise to hippuric acid in the body, they increase the acidity of the urine (see p. 1097).

‡ Ripe olives are soaked in alkali during their manufacture, and the alkaline content is apt to be high.

V. MAINTENANCE OF ANIMALS FOR NUTRITION EXPERIMENTS

Introduction. Laboratory animals should be housed in well-ventilated rooms free from drafts in order to avoid colds or the blowing around of finely divided dietary dust. Rooms should be diffusely lighted during the day and darkened at night, the alternating periods of light and darkness being regular from day to day. Exposure of rats (or chicks) to direct sunlight, even though filtered through window glass, must be avoided in vitamin D assay work. Rats are nocturnal animals and excessive noise or activity during the day interferes with their normal habits.

Air-conditioning of rooms is desirable but not essential except where extreme temperatures would otherwise occur. The optimum environmental temperature for small animals is 24 ± 2° C. Provision should be made for screening windows and for frequent washing and flushing of walls and floors, which should be sealed against vermin and wild rodents.

Extermination of insects such as roaches, flies, or bedbugs should be commenced as soon as they are observed. With sufficient attention and use of modern insecticides, infestation can be effectively eradicated.

Flies and Roaches. If the room can be cleared of animals, spray[60] thoroughly with a potent commercial insecticide such as those containing pyrethrum,[61] lethane, DDT, methoxychlor, or piperonylbutoxide. Seal room for several hours or overnight. Air thoroughly before returning animals.

If animals cannot be removed from the room, apply the insecticide by means of a soft brush to walls and floors (especially in corners, cracks, and seams), cage racks, and other equipment except the cages themselves.

Bedbugs. Paint walls, crevices and equipment with a 5 per cent solution of DDT in kerosene. One application may suffice to eradicate a severe infestation; if not, repeat after six weeks.

Excreta should be removed at least every other day. Paper rolls or thick layers of newspaper under racks of raised-bottom cages facilitate this operation. Hot tin-dipped galvanized wire cages are preferred. These should be cleaned once every week or two depending upon the number of occupants per cage. Cages should be scraped, scrubbed in hot soap solution or soaked in hot trisodium phosphate solution and finally rinsed thoroughly in hot, then cold water.

Rats.[62] Selectively bred albino or piebald rats are the animals *par*

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[60] Hand spraying is not nearly as efficient as the use of an electric power spray.

[61] Many insecticidal sprays contain an insufficient concentration of active pyrethrins. A very effective product is Fumol, made by The Fumol Corporation, Long Island City, N. Y.

[62] For handbooks on the care of laboratory animals and their use in experimental work, see Haberland: *Die operative Technik des tierexperimentes*, Berlin, Springer, 1926; Pittenger: *Biologic Assays*, 2d ed., Philadelphia, The Blakiston Company, 1928; Munch: *Bioassays*, Baltimore, The Williams & Wilkins Co., 1931; *Manual of Biological Assaying*, Philadelphia, J. B. Lippincott Co., 1937; Main: *The Care of a Small Rat Colony*, St. Louis, C. V. Mosby Co., 1939; Farris and Griffith: *The Rat in Laboratory Investigation*, 2nd ed., Philadelphia, J. B. Lippincott Co., 1949; Farris: *The Care and Breeding of Laboratory Animals*, New York, J. Wiley & Sons, Inc., 1950.

See also Greenman and Duhring: *The Breeding and Care of the Albino Rat*, Philadelphia, Wistar Institute, 1931. Extensive biometric data may be found in Donaldson: *The Rat*, 2d ed., Philadelphia, Wistar Institute, 1924; Hunt: *A Laboratory Manual of the Anatomy* *excellence* for nutrition experiments.[63] Black rats are of course more suitable for study of achromotrichia although white rats develop a condition described as "rusting," comparable to graying of black fur. In their dietary habits rats, like man, are omnivorous. Their period of growth (see Fig. 294) extends over approximately 300 days and their life span is about three years. Thus they live through their cycle at about 30 times the rate of the human species, however, compared with other species the rate of prepubertal growth of man is slower. Rats multiply rapidly and the large litters which they produce permit of a high degree of experimental control. They require but little food and are hence economical to use. For studies of certain nutritional deficiencies to which the rat is immune, notably scurvy, other species of animals must be employed. Variation in species requirements (rat *vs.* chick) was partly responsible for the discovery of the multiple nature of vitamin D and of the vitamin B complex.

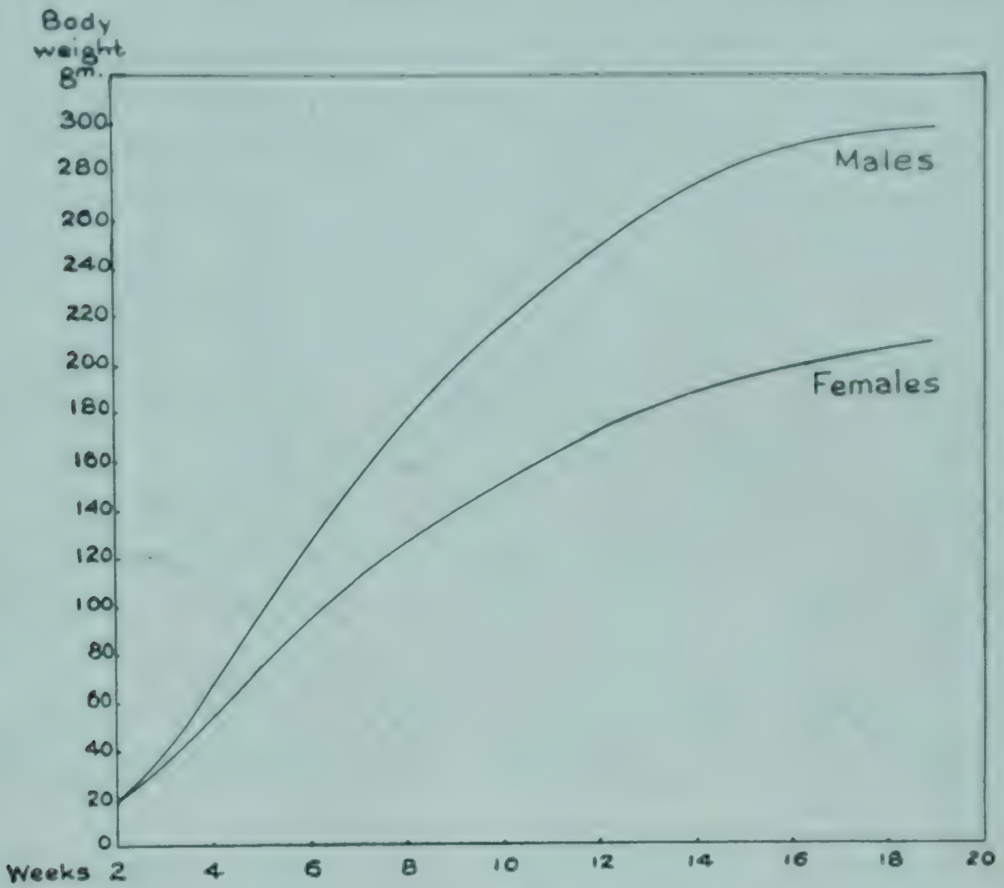

FIG. 294. NORMAL GROWTH CURVES OF MALE AND FEMALE ALBINO RATS.[64]

Food Research Laboratories' Breeding Colony.

Rats attain sexual maturity at about 75 days, but should not be mated for breeding purposes until about 100 days of age. One male may be

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of the Rat, New York, The Macmillan Co., 1925. See also Howell: *Anatomy of the Wood Rat*, Baltimore, The Williams & Wilkins Co., 1926, and especially Greene: *Anatomy of the Rat*, Philadelphia, American Philosophical Society, 1935.

[63] For uniform results in vitamin assay work animals should be bred and reared on stock diets of constant composition, preferably under the direct control of the assayer.

[64] These curves are based on 1946 data. Currently (1954) average weights are 20–30 g. higher from about 16 weeks.

A linear transformation of the normal growth curve can be effected by plotting the log weight vs. reciprocal of the age according to the method of Zucker *et al.* (Zucker, Hall Young and Zucker: *J. Nutrition*, **22**, 123 (1941); Zucker and Zucker: *J. Gen. Physiol.*, **25**, 445 (1942); Gray and Addis: *Am. J. Physiol.*, **153**, 35 (1948)). This procedure facilitates comparison of growth data since it permits expression of the responses in terms of the slope of the line and the extrapolated asymptotic (theoretical maximum) weight.

caged with as many as three females at one time. Pregnant females should be isolated before litters are cast, unless only one female occupies the cage with the male. The gestation period is about 22 days. The young are usually weaned when three to four weeks old. If the number of pups in a litter exceeds 8, it should be reduced to that number to protect the mother and at the same time produce sturdier rats. If litters are cast in wire-mesh cages, the mesh should be less than ½ inch and shredded paper or cellophane should be furnished for bedding or nesting.

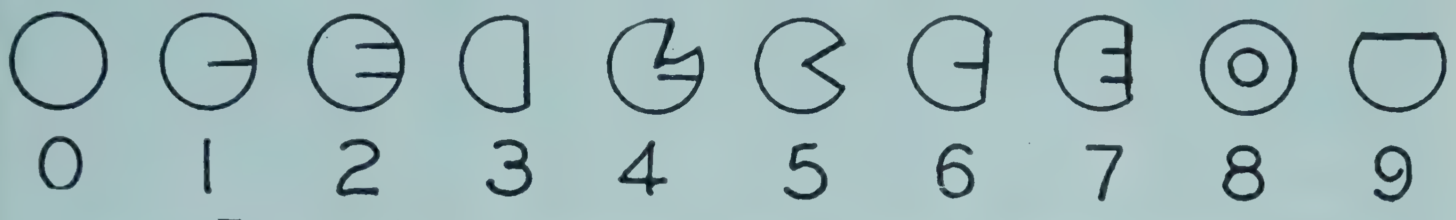

FIG. 295. SCHEME FOR NUMERICAL EARMARKING OF RATS.

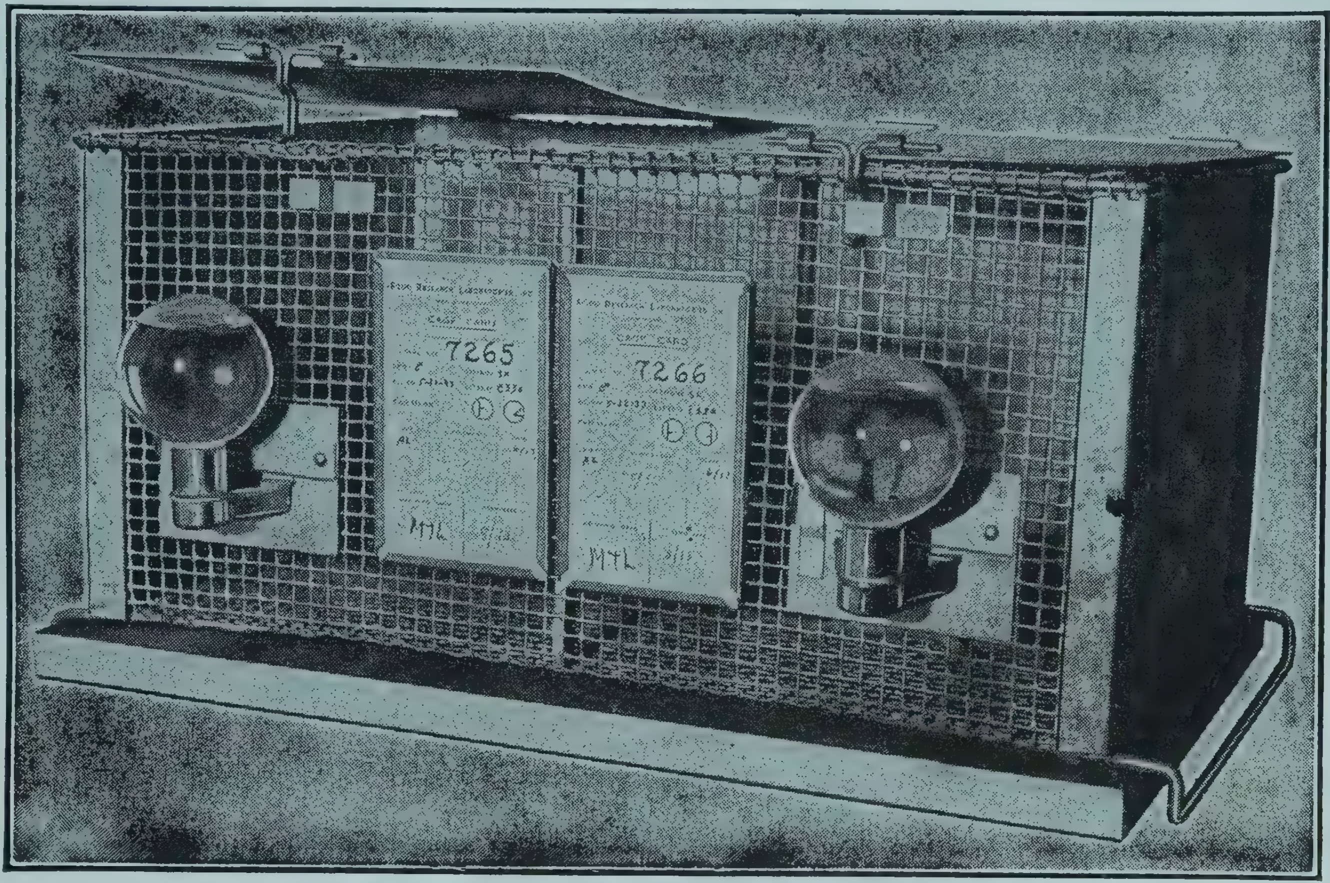

FIG. 296. INDIVIDUAL BREEDING CAGE FOR RATS.

With Hendryx water fountains, removable partition, double lid, positive action catch, and sliding tray. The front, bottom, and back are constructed of a continuous sheet of wire mesh.

Constructed to specifications of Food Research Laboratories, Inc., by Norwich Wire Works, Norwich, N. Y.

Rats may be marked by staining the fur with dyes (e.g., methylene blue or picric acid), by means of an ear tattoo-punch,[65] or by earmarking by means of combinations of nine types of cuts (slits, V-shaped notches, or holes) in both ears thus numbering them permanently over a range from 0 to 99. (Fig. 295.)

For breeding and stock purposes in small colonies the cage illustrated in Fig. 296 may be employed; for larger laboratories racks of suspended drawer-type cages are most suitable. These may be equipped to carry outside water bottles and to provide for paper rolls for the collection and removal of excreta (see Fig. 297), although stainless-steel trays and

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[65] Keeler: *Science*, **12**, 205 (1940).

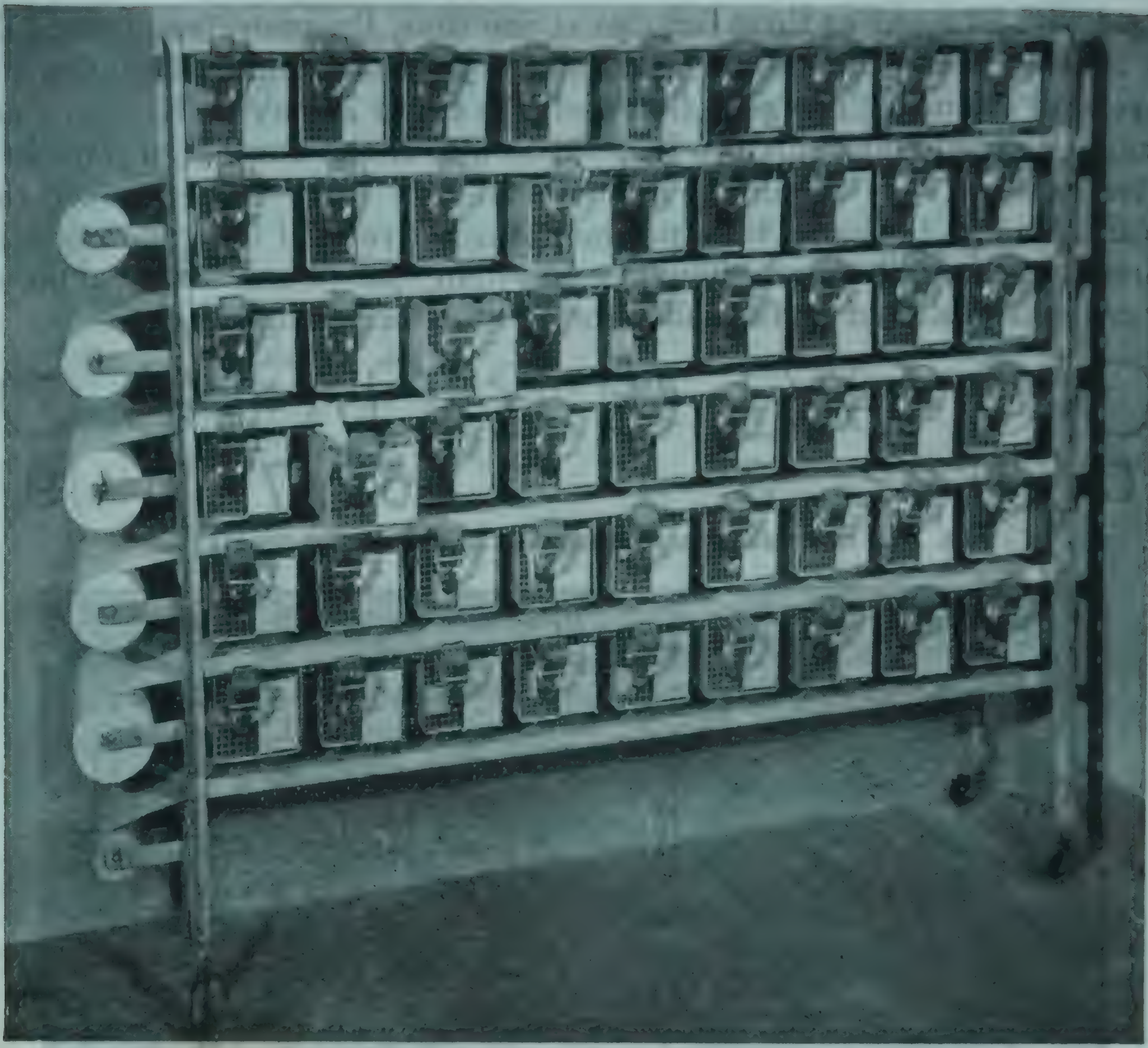


FIG. 297. BATTERY OF RAT CAGES OF THE SUSPENSION TYPE.
Manufactured by Norwich Wire Works, Norwich, N. Y.

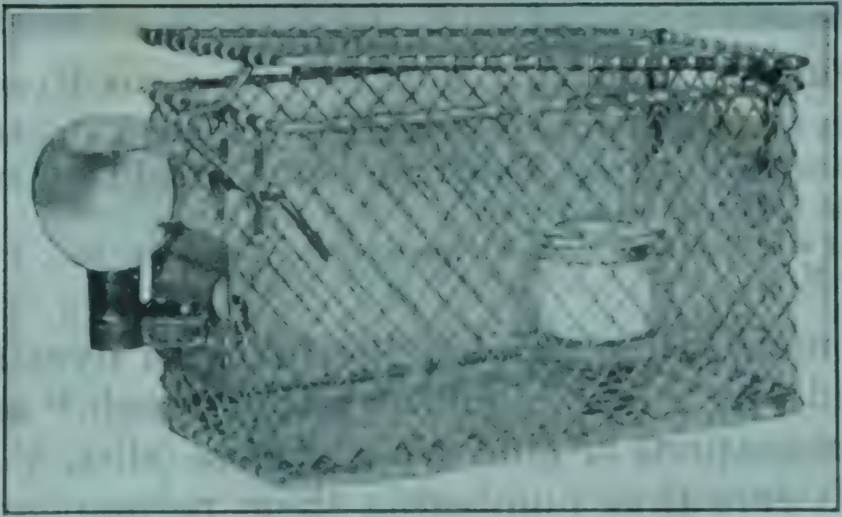
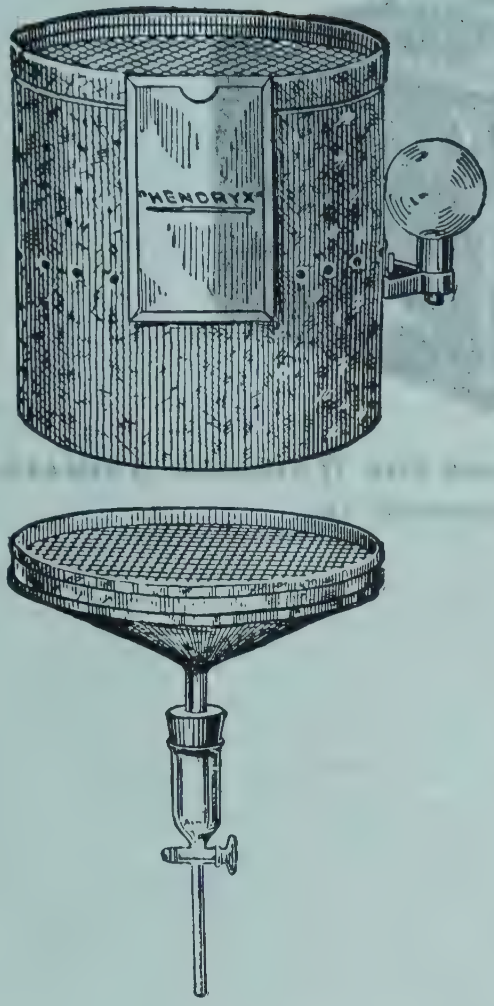


FIG. 298. INDIVIDUAL RAT CAGE, OF ALL-WIRE CONSTRUCTION.

With Hendryx water fountain, positive action catch. Nonscatter feed cup made of 4-oz. ointment jar with plastic cover perforated with $1\frac{1}{4}''$ hole.

Cage constructed to specifications of Food Research Laboratories, Inc., by Norwich Wire Works, Norwich, N. Y. Feed cup supplied by Armstrong Cork Co., Lancaster, Pa.



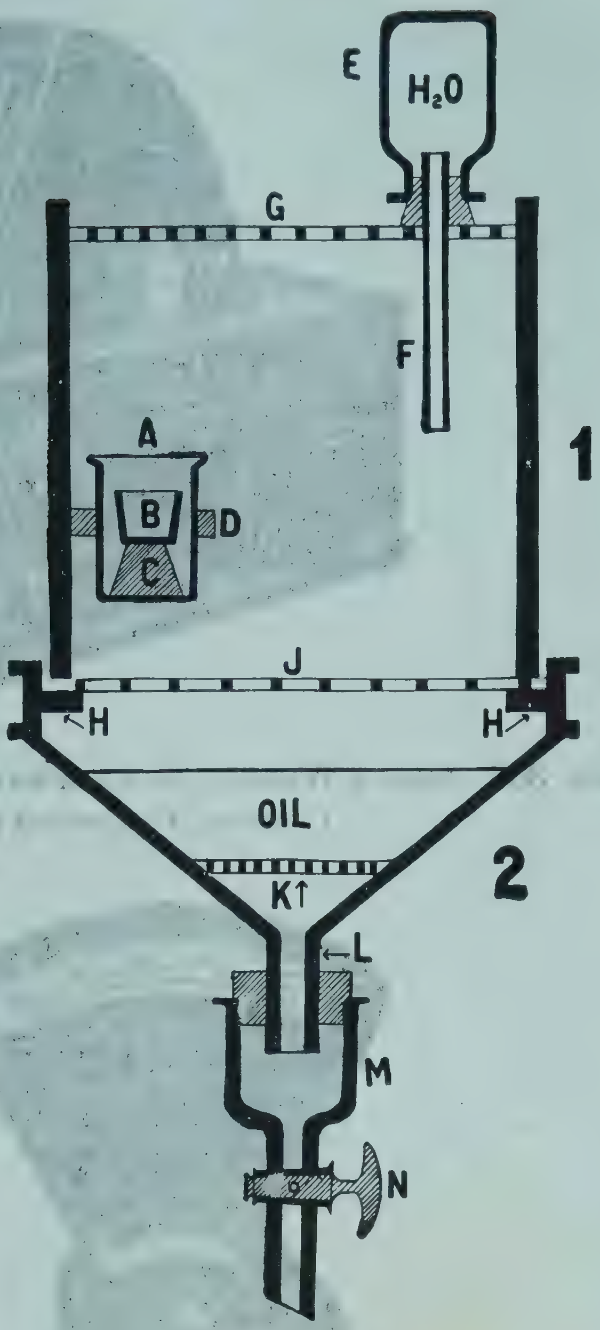

E
H₂O
G
F
1
A
B
D
C
J
←H
H→
OIL
2
K↑
←L
M
N

FIG. 299. (*Left*) METABOLISM CAGE FOR SMALL ANIMALS.
FIG. 300. (*Right*) CROSS SECTION OF A METABOLISM CAGE.

Courtesy, Levine and Smith: *J. Lab. Clin. Med.*, 11, 168 (1925).

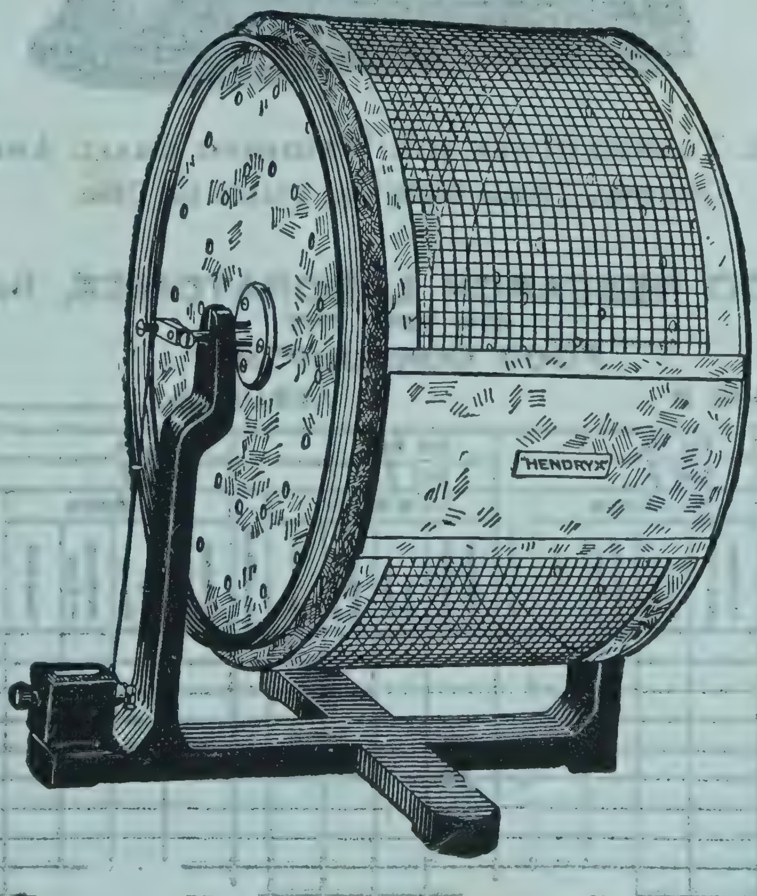


FIG. 301. VOLUNTARY ACTIVITY CAGE FOR RATS.

As used at Yale University.

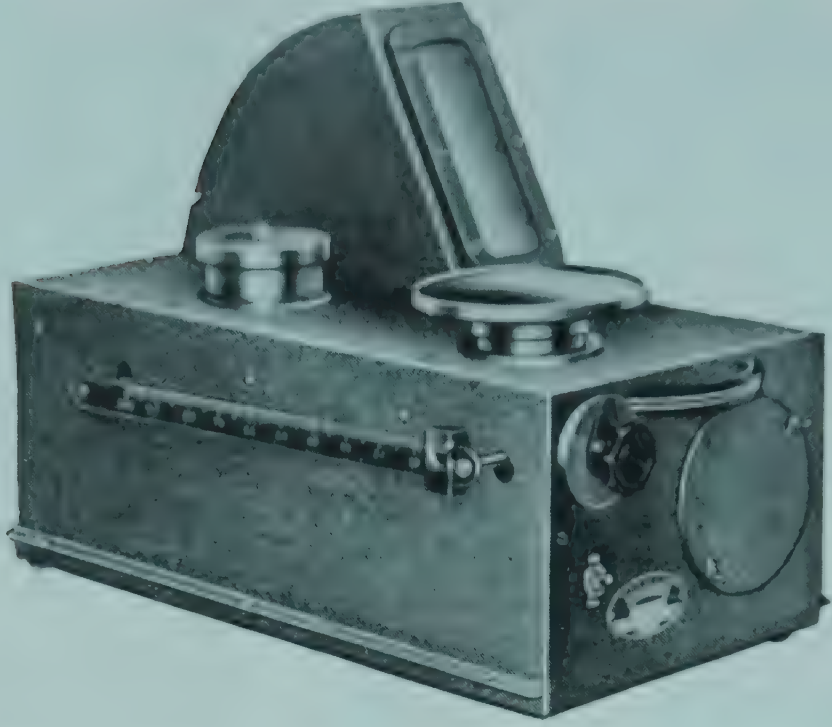


FIG. 302. EXACT WEIGHT SHADOGRAPH SCALE FOR WEIGHING ANIMALS.
Courtesy, Underwood and Underwood, Inc.

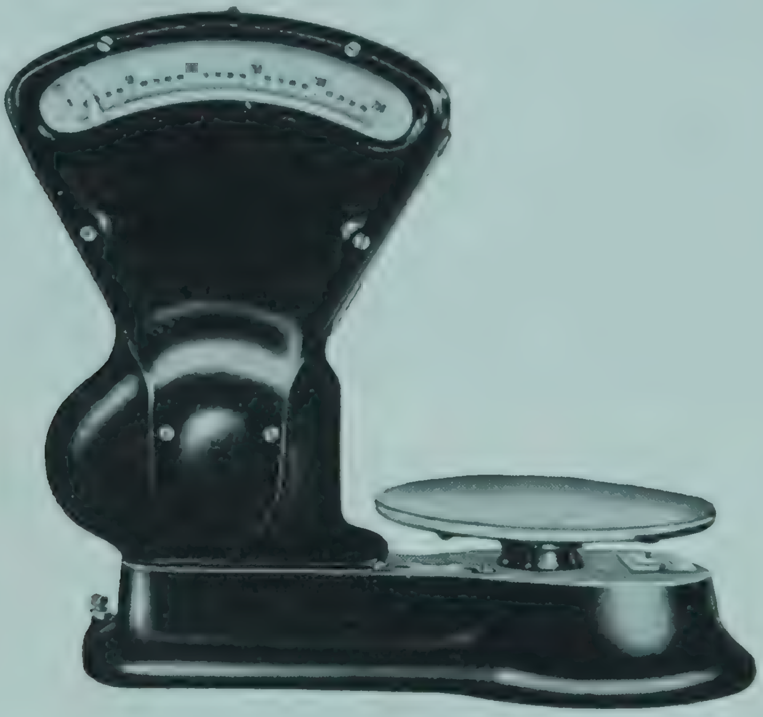
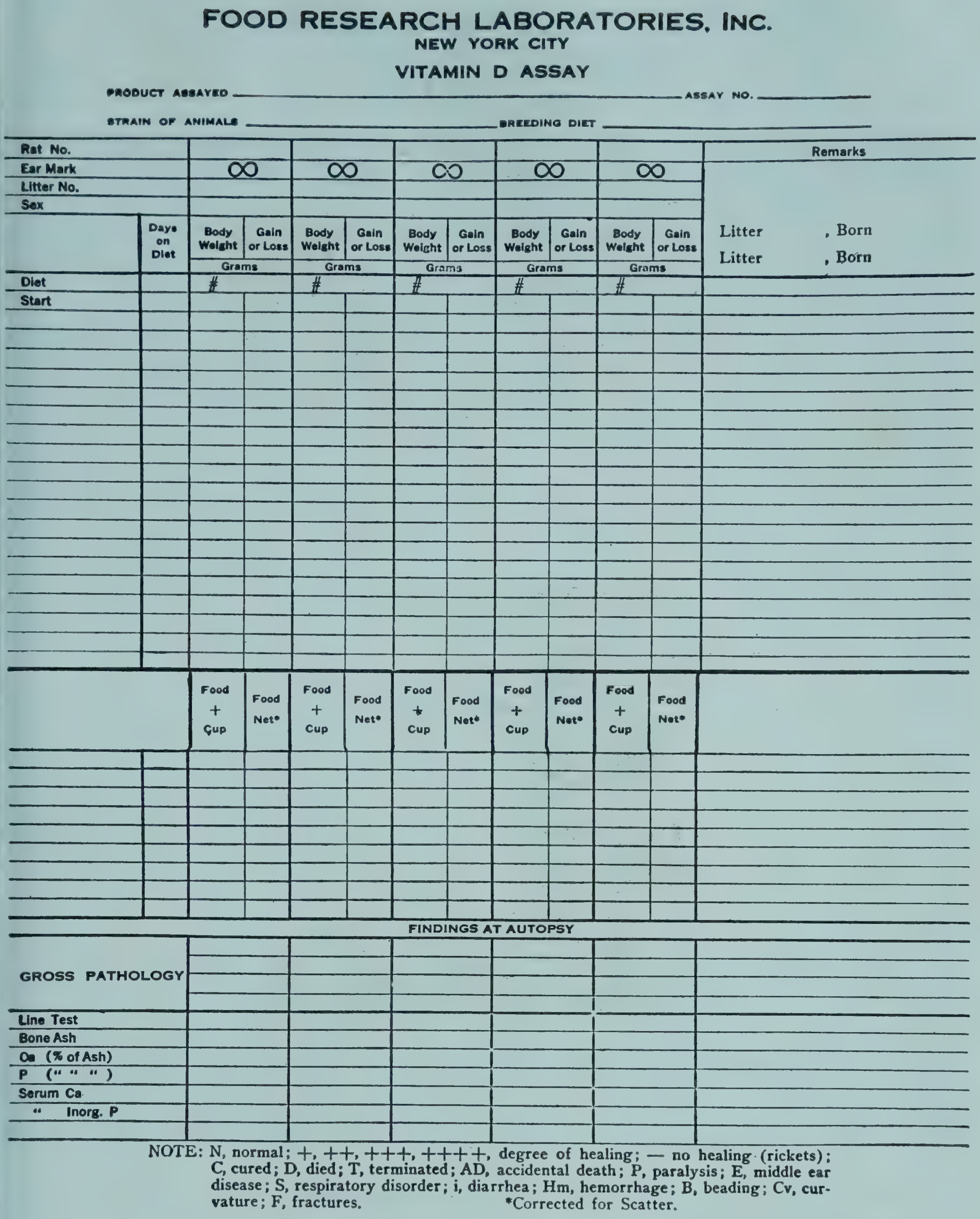


FIG. 303. TOLEDO SCALE FOR WEIGHING SMALL ANIMALS.
Courtesy, Toledo Scale Co., Toledo 12, Ohio.

FOOD RESEARCH LABORATORIES, INC.
NEW YORK CITY
FOOD CONSUMPTION
VITAMIN D **ASSAY No.**__________

Date	Rat No.					Rat No.					Rat No.					Rat No.					Rat No.				
	Diet #					Diet #					Diet #					Diet #					Diet #				
	Wt. in Grams					Wt. in Grams					Wt. in Grams					Wt. in Grams					Wt. in Grams				
	Container plus Food	Food (Gross)	Scattered in Cage	Food Net	No. of Days	Container plus Food	Food (Gross)	Scattered in Cage	Food Net	No. of Days	Container plus Food	Food (Gross)	Scattered in Cage	Food Net	No. of Days	Container plus Food	Food (Gross)	Scattered in Cage	Food Net	No. of Days	Container plus Food	Food (Gross)	Scattered in Cage	Food Net	No. of Days

FIG. 304. FOOD CONSUMPTION CHART.



FOOD RESEARCH LABORATORIES, INC.
NEW YORK CITY
VITAMIN D ASSAY

PRODUCT ASSAYED ______ ASSAY NO. ______

STRAIN OF ANIMALS ______ BREEDING DIET ______

Rat No.												Remarks
Ear Mark		∞		∞		∞		∞		∞		
Litter No.												
Sex												
	Days on Diet	Body Weight	Gain or Loss	Body Weight	Gain or Loss	Body Weight	Gain or Loss	Body Weight	Gain or Loss	Body Weight	Gain or Loss	Litter , Born Litter , Born
		Grams		Grams		Grams		Grams		Grams		
Diet		#		#		#		#		#		
Start												
		Food + Cup	Food Net*	Food + Cup	Food Net*	Food + Cup	Food Net*	Food + Cup	Food Net*	Food + Cup	Food Net*	

FINDINGS AT AUTOPSY

GROSS PATHOLOGY						
Line Test						
Bone Ash						
Ca (% of Ash)						
P (" " ")						
Serum Ca						
" Inorg. P						

NOTE: N, normal; +, ++, +++, ++++, degree of healing; — no healing (rickets); C, cured; D, died; T, terminated; AD, accidental death; P, paralysis; E, middle ear disease; S, respiratory disorder; i, diarrhea; Hm, hemorrhage; B, beading; Cv, curvature; F, fractures. *Corrected for Scatter.

FIG. 305. EXAMPLE OF DATA SHEET FOR VITAMIN ASSAY.

thick layers of newspaper are in some respects preferable. When they are ready for experiments the rats should be transferred to individual cages of the type illustrated in Fig. 298. These cages are of all-wire construction and have false bottoms to minimize access to excreta.[66] They are easily sterilized.

Specially prepared diets should be fed in a nonscatter type of food cup to permit the collection of more accurate data on food consumption. Suitable for this purpose is a 3- or 4-oz. screw-cap ointment jar with a

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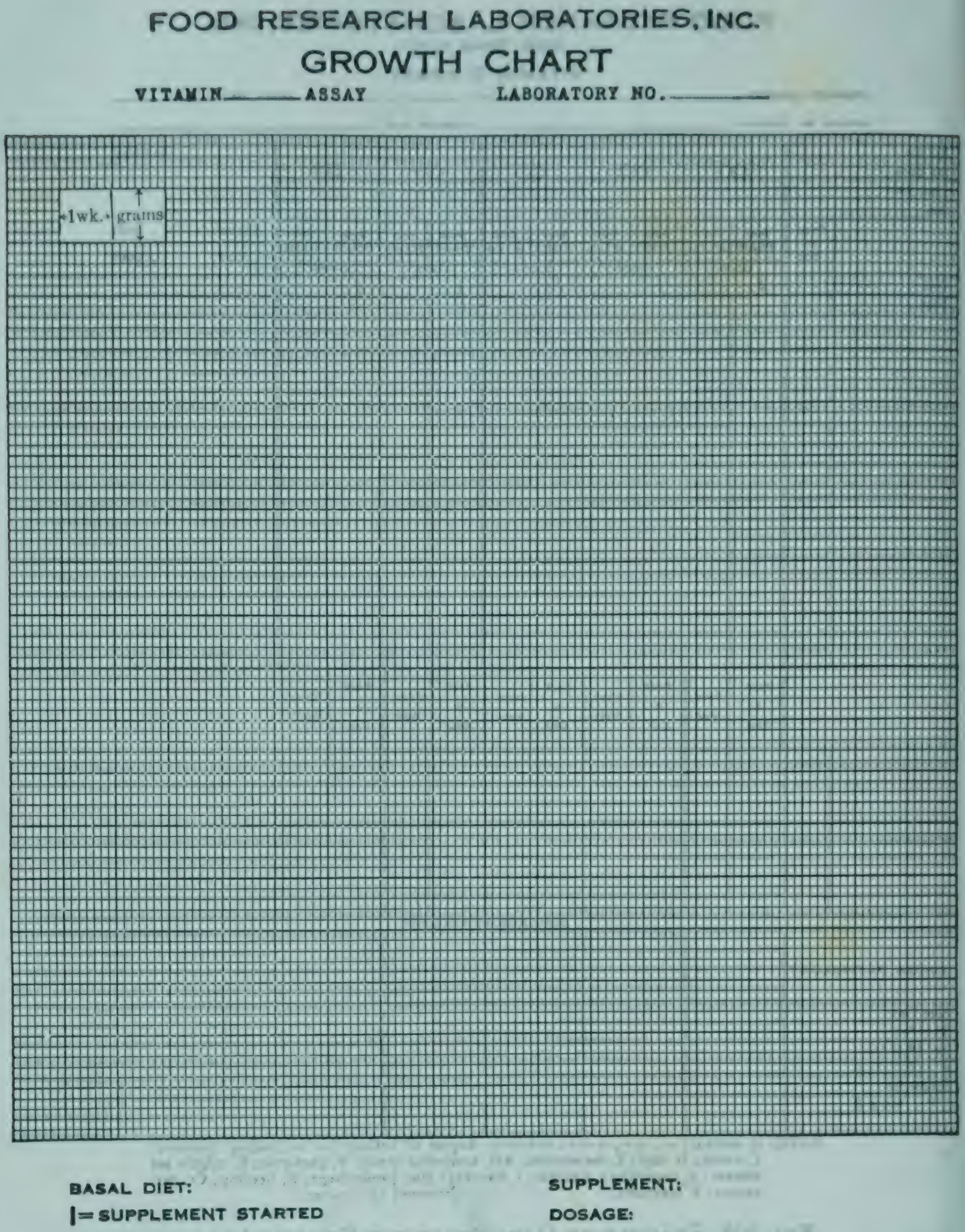
[66] Cages and accessories may be obtained from the Norwich Wire Works, Norwich, New York; the Geo. H. Wahmann Mfg. Co., Baltimore, Md.; or the A. B. Hendryx Co., New Haven, Connecticut.



FOOD RESEARCH LABORATORIES, INC.
GROWTH CHART
VITAMIN ______ ASSAY LABORATORY NO. ______

BASAL DIET:
| = SUPPLEMENT STARTED
SUPPLEMENT:
DOSAGE:

FIG. 306. GROWTH CURVE CHART.

hard plastic cap through which a 30-mm. hole is cut. (See food cup inside cage in Fig. 298.) Dietary supplements may be weighed or measured in small opal glass cups (rouge pots). Oils or liquid supplements are preferably fed directly into the mouth from a tuberculin syringe fitted with a needle (No. 16 or 18) ground to a smooth, blunt tip. For nutritional balance experiments, in which separation and collection of urine and feces are necessary, cages of the type shown in Figs. 299 and 300 may be used. The activity cages (Fig. 301) may be employed as illustrated for studies in voluntary activity, or may be driven by a pulley to produce enforced activity.

Rats may be weighed on a spring balance (Chatillon or Hanson), but for more exact work an Exact Weight or Toledo scale of 250- 500-, or 1000-g. capacity, sensitive to 1 g. should be used. (See Figs. 302 and 303.) These balances are also suitable for weighing food cups. Records are kept of the weekly or semiweekly weighings on form sheets such as are illustrated in Figs. 304 and 305. A modified type of growth chart (Fig. 306) in which the time coordinates (abscissae) are divided into weeks and days has proved very useful.

Breeding and Stock Diets. When rats are reared for purposes not requiring careful control of their nutrition, they may be fed a mixed diet consisting of clean table scraps, bread and milk, or dog biscuit[67] supplemented with milk or fresh vegetables such as carrots or lettuce, once or twice a week. Specially prepared rations for rats and other laboratory animals are available on the market and have proved quite satisfactory.[68] Mixed poultry rations (growing mash) or calf meal may also be used. For careful nutritional research it is necessary to use a uniform stock diet whose efficiency has been established through several generations of rats. It is desirable that rats reared for investigations on the fat-soluble vitamins be fed diets which limit or control their reserves of the vitamins in question.

1. FOOD RESEARCH LABORATORIES' RAT STOCK DIET[69]

Whole wheat	32	Vitamin B-complex mixture[70]	1
Yellow corn	16	Vitamins in oil[71]	1
Nonfat milk solids	20	Cellulose powder	1
Hydrogenated cottonseed oil	15	NaCl	0.5
Meat meal (65% protein)	10	$MnSO_4 \cdot H_2O$	0.02
Alfalfa (kiln-dried)	2	Pork liver (vacuum-dried)	0.48
Yeast (dried)	1		

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[67] The dog biscuits used should be of known nutritional quality as there is a great variation among them, some being incapable even of supporting normal growth. Certain brands of pet foods are available in the form of unpelleted mixtures or mashes.

[68] Ralston Purina Co., St. Louis, Mo., supplies such rations in the form of compressed "checkers" and meal (Purina Laboratory Chow for rats and mice, and special chows for guinea pigs, rabbits, dogs, etc.). Similar rations are available through Rockland Farms, New City, New York.

[69] Based originally on the Sherman Diet B (*J. Biol. Chem.*, **53**, 49 (1922); **60**, 5 (1924)), this diet has been modified several times to improve breeding results. The present composition brings it into reasonably close agreement with the Department of Agriculture data for U.S. food consumption, particularly with respect to the distribution of the major categories of foods (grains and cereals, meat and meat products, milk and dairy products). (Oser, B. L., and M. Oser: Unpublished data.)

[70] Vitamin B-complex mixture, 1 g. contains:

Thiamine	0.6 mg.	Choline chloride	200 mg.
Riboflavin	1.2 mg.	Liver concentrate 1:20	25 mg.
Pyridoxine	0.4 mg.	Biotin	1 μg.
Niacin	5.0 mg.	Folic acid	1 μg.
Calcium pantothenate	4.0 mg.	Cyanocobalamin	1 μg.
p-Aminobenzoic acid	2.5 mg.	Cellulose powder q.s.	1 g.
Inositol	100 mg.		

[71] Vitamins in oil, 1 g. contains:

Vitamin A	200 units
Vitamin D	20 units
α-Tocopherol	12 mg.
Menadione	100 μg.
Cottonseed oil q.s.	1 g.

2. Bills' Modification[72] of the Steenbock Stock Diet

Yellow corn	57
Dried whole milk	25
Linseed oil meal	12
Crude casein	3.7
Alfalfa leaf meal	1.5
Iodized table salt	0.4
Calcium carbonate	0.4

3. A. D. M. A. Breeder Diet

Wheat meal (entire kernel)	33.0
Yellow corn meal (entire kernel)	34.0
Whole milk powder	21.0
Old process linseed oil meal	7.0
Alfalfa leaf flour (green)	2.0
Pork liver (vacuum dried)	2.0
Calcium carbonate	0.5
Sodium chloride	0.5
Lettuce or spinach	about 5 g. daily

4. F. R. L. Vitamin-Restricted Diet[73]

Wheat meal (entire kernel)	37.0
Yellow corn meal (entire kernel)	37.0
Whole milk powder[74]	22.0
Dried yeast[74]	1.0
Dried pork liver	0.2
Dried alfalfa	2.0
Calcium carbonate	0.5
Sodium chloride	0.5
Manganese sulfate	0.02
Liver concentrate (Wilson 1:20)	0.1

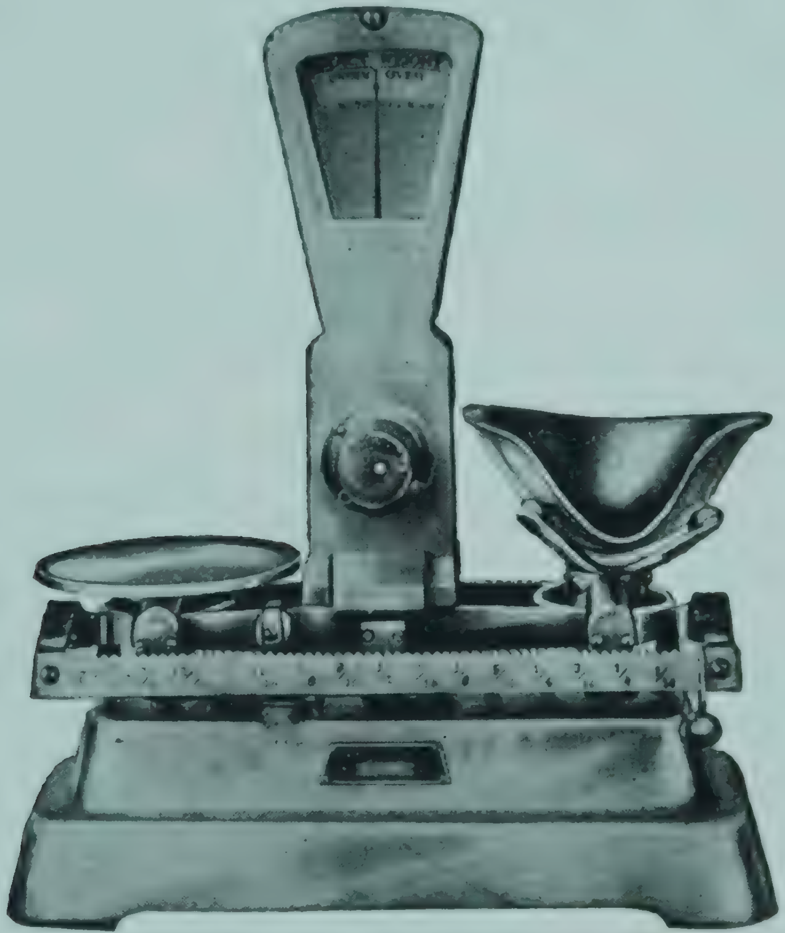

FIG. 307. EXACT WEIGHT SCALE FOR PREPARATION OF EXPERIMENTAL DIETS.
Courtesy, Underwood and Underwood, Inc.

Typical diets which have proved successful are shown on p. 1373-4. These may be prepared by means of scales of the type illustrated in Fig. 307.

Salt Mixtures. Synthetic diet mixtures used in nutritional research on rats include special combinations of inorganic salts whose composition is based on the analysis of their milk[75] or urine. The original Osborne-Mendel and McCollum-Davis salt mixtures, given below, have been modified to include readily available commercial forms of the chemicals employed (see p. 1262). The modification of the Osborne-Mendel mixture by Hawk and Oser includes salts which yield a similar stoichiometric mixture but, by avoiding acids and carbonates, eliminates the need for evaporation and dehydration.

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[72] Bills, Honeywell, Wirick, and Nussmeier: *J. Biol. Chem.*, **90**, 619 (1931). Lindow, Peterson, and Steenbock: *J. Biol. Chem.*, **84**, 419 (1929).

[73] In some laboratories a more highly restricted diet may be used with safety in vitamin A work. For this purpose skim milk powder may be substituted for whole milk powder.

[74] Not irradiated or fortified with vitamin D.

OSBORNE-MENDEL SALT MIXTURE[76]

$CaCO_3$	134.8	H_3PO_4	103.2	Fe citrate $1\frac{1}{2}$ H_2O	6.34
K_2CO_3	141.3	HCl	53.4	NaF	0.062
$MgCO_3$	24.2	H_2SO_4	9.2	$KAl(SO_4)_2$	0.0245
Na_2CO_3	34.2	Citric acid + H_2O	111.1	$MnSO_4$	0.079
				KI	0.020

All amounts are given in grams. The acids are added to the carbonates and ferric citrate, and stock solutions of the other salts are then added in proper amount. After effervescence is completed the mixture is dried at 90° to 100° C. and ground to a fine powder. These amounts of phosphoric, hydrochloric and sulfuric acids are equivalent to 71, 121, and 5.2 ml., respectively, of the concentrated acids. See also p. 1262.

HAWK-OSER SALT MIXTURE NO. 3[77]

Ca Citrate·$4H_2O$		308.2
$Ca(H_2PO_4)_2 \cdot H_2O$		112.8
K_2HPO_4		218.7
KCl		124.7
NaCl		77.0
$CaCO_3$		68.5
$3\,MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$		35.1
$MgSO_4$ anhydrous		38.3
Fe NH_4 Citrate U.S.P.	91.41	16.7
$CuSO_4 \cdot 5H_2O$	5.98	
NaF	0.76	
$MnSO_4 \cdot 2H_2O$	1.07	
$KAl(SO_4)_2 \cdot 12H_2O$	0.54	
KI	0.24	
	100.00	1000.00

HUBBELL, MENDEL, AND WAKEMAN SALT MIXTURE[78]

$CaCO_3$	543.0
$MgCO_3$	25.0
$MgSO_4$	16.0
NaCl	69.0
KCl	112.0
KH_2PO_4	212.0
$FePO_4 \cdot 4H_2O$	20.5
KI	0.08
$MnSO_4$	0.35
NaF	1.00
$Al_2(SO_4)_3K_2SO_4$	0.17
$CuSO_4$	0.90

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[75] An analysis of rat milk compared with human and cow's milk is given by Cox and Mueller: *J. Nutrition,* **13,** 249 (1937).

[76] Osborne and Mendel: *J. Biol. Chem.,* **15,** 317 (1913).

[77] Hawk and Oser: *Science,* **74,** 369 (1931). Stoichiometrically the approximate equivalent of Osborne-Mendel's but made by mixing dry salts. Adopted as Salt Mixture No. 1 in the U.S.P. XIV Vitamin A-free Diet (modified, 1953).

[78] Hubbell, Mendel, and Wakeman: *J. Nutrition,* **14,** 273 (1937). Salts used are "reagent" or c.p. grade. *Two per cent of this mixture may be used in place of 4 per cent of the Osborne-Mendel or Hawk-Oser mixtures.*

McCOLLUM-DAVIS SALT MIXTURE
No. 185[79]

Ca lactate	35.15
$Ca(H_2PO_4)_2H_2O$	14.60
K_2HPO_4	25.78
$NaH_2PO_4 \cdot H_2O$	9.38
NaCl	4.67
$MgSO_4$ (anhydrous)	7.19
Fe citrate	3.19

STEENBOCK SALTS 40[80]

NaCl	233.6
$MgSO_4 \cdot 7H_2O$	246.0
$Na_2HPO_4 \cdot 12H_2O$	358.0
K_2HPO_4	696.0
$CaHPO_4 \cdot 2H_2O$	698.0
$Ca(C_3H_5O_3)_2 \cdot 5H_2O$	154.0
$Fe(C_6H_5O_7)_2 \cdot 6H_2O$	59.8
KI	1.6

Guinea Pigs (Cavies). The most suitable guinea pig for nutrition work is the English short smooth-haired variety, whose manifold coloring facilitates identification. Not all pure white guinea pigs are albinos since they may have dark-colored instead of pink eyes.

Guinea pigs must be housed away from drafts in dry cages, preferably with false bottoms although beds of sawdust or shavings are satisfactory. Dry hay should be provided at all times. They are herbivorous and may be fed clean hay and grain (oat meal, bran, wheat middlings, etc.) supplemented with fresh vegetables (carrots, beets, alfalfa, lettuce, celery, etc.). Moist vegetables must be sound and wholesome because guinea pigs will reject spoiled food. Diets containing fats (e.g., codliver oil) must not be fed when rancid. The addition of dried yeast to the grain mixture is frequently desirable. Specially prepared diet mixtures for guinea pigs are available commercially.[81] Water should be fed sparingly when fresh vegetables are supplied.

Most investigators purchase guinea pigs on the market, since their nutritional history is seldom of importance. However, they should be purchased from a reputable breeder[82] since they are prone to carry pulmonary and other infections when raised or shipped under adverse conditions. Guinea pigs breed about once every three or four months, the gestation period being from 65 to 70 days. Mating should commence not before the age of three months. The lactation period should last about two to three weeks.

White Mice. The white mouse has been employed with some success in studies in nutrition.[83] Advantages of the mouse include a smaller food requirement, more rapid growth, and frequency of reproduction. The dietary needs of the mouse are similar to those of the rat although a somewhat higher protein content is recommended.

Advantages of the rat over the mouse for nutritional work include the fact that the rat has been more extensively studied, thus affording more norms for comparison; also it is hardier than the mouse, and thus not so prone to contract minor illnesses or to be adversely influenced by changes in temperature, etc.

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[79] McCollum and Davis: *J. Biol. Chem.*, **33**, 55 (1918).

[80] Steenbock and Nelson: *J. Biol. Chem.*, **56**, 355 (1923).

[81] See footnote 68, p. 1373.

[82] See footnote 63, p. 1366.

[83] Beard: *Am. J. Physiol.*, **75**, 645, 658, 668, 682 (1926). Bing and Mendel: *J. Nutrition*, **2**, 49 (1929). For ophthalmia studies see Pomerene and Beard: *Am. J. Physiol.*, **92**, 282 (1930). Woolley: *J. Biol. Chem.*, **139**, 29 (1941) used the mouse for inositol studies and Nielson and Black: *J. Nutrition*, **28**, 203 (1944) for the study of biotin and pteroylglutamic acid deficiencies.

It has been claimed that mice require vitamin C for normal development.[84]

Dogs.[85] The feeding of a purely "synthetic" food mixture for long periods, in metabolism tests using dogs as experimental animals, is a procedure for the successful accomplishment of which investigators in metabolism have long striven. It remained for Cowgill to solve this important problem in dietary technique. His work was an extension of that initiated by Karr.[86]

The principles upon which the procedure is based are that the diet consist of a mixture of isolated food substances which supply the body with all factors necessary for proper nutrition, except a single dietary factor; and that the variable nutritive factor shall be administered apart from the diet mixture. In this discussion vitamin B (complex) is regarded as this variable nutrition factor.

In a test where vitamin B is the variable factor, the diet[87] should contain (1) *protein*, proper in kind and amount;[88] (2) *carbohydrate*,[89] and *fat*[90] to furnish satisfactory energy; (3) *mineral salts*,[91] proper in kind and

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[84] Kleiner and Tauber: *Food Research*, **1**, 399 (1936).

[85] Cowgill: *J. Biol. Chem.*, **56**, 725 (1923). This procedure has been used at Yale University for periods of five months and over with excellent results.

[86] Karr: *J. Biol. Chem.*, **44**, 255 (1920).

[87] *Calculation of the Diet.* Construct the diet on a kg. of body weight basis, or form what might be called a *kilo unit of food.* Dogs weighing 7 kg. and over require for the maintenance of body weight between 70 and 80 Calories per kg. per day. 80 Calories permits *maintenance* in adult animals and allows a slight increase of body weight in growing dogs. Dogs weighing considerably less than 7 kg. require slightly more energy intake in order to maintain their body weight; this fact, however, does not invalidate the choice of 80 Calories as the kilo unit quantity. Any variation in energy intake from this figure due to the size of the animal will not seriously affect the intake of nitrogen and salt mixture if liberal quantities such as 0.8 g. N and at least 0.2 g. salt mixture are furnished with every kilo unit of food. For further discussion of the basis for this diet consult the original paper.

[88] This may be *casein*, *extracted meat* (from the Valentine Meat Juice Co., Richmond, Va.) or *commercial coagulated egg albumin.* Casein is to be preferred.

[89] *Sucrose* is a desirable source of carbohydrate; pure starch or dextrin make the diet too pasty when moistened by saliva. Whole cereals, however, are suitable.

[90] See footnote 92, p. 1378.

[91] Cowgill suggests the use of one of the following salt mixtures:

SALT MIXTURE (KARR, 1920)	
NaCl	10 g.
Ca lactate	4 g.
Mg citrate	4 g.
Fe citrate	1 g.
Iodine in KI (Lugol's solution)	3 drops

SALT MIXTURE (COWGILL)	
NaCl	38.0
Mg citrate	32.5
KH_2PO_4	12.2
$CaHPO_4 \cdot 2H_2O$	7.8
KCl	7.0
Fe citrate	1.8
KI	0.5

Karr's salt mixture, when fed with bone ash as a source of phosphate on the basis of 0.2 g. and 0.4 g., respectively, per kilo unit of food has given successful results during periods lasting over five months.

Where 0.4 g. of agar-agar per kilo unit is used as a source of roughage, Cowgill's salt mixture may be used on the basis of 0.3 g. per kilo unit of food. It is supposed to furnish the dog the various essential inorganic substances equal to or slightly in excess of that eliminated through the kidney by a normal organism during 24 hours, these amounts being expressed in terms of g. eliminated per kilo of body weight per day; 0.3 g. of this mixture amount; (4) sufficient *vitamin A*;[92] (5) *roughage*,[93] and (6) *water*.[94] The energy requirement as shown in the table of the "kilo unit" below is somewhat high for dogs whose activity is restricted, e.g., in metabolism cages. In such cases it is advisable to reduce the calories to 60–70 per kg. body weight by substituting sucrose for lard. The vitamin B (the variable factor) should be fed *separately from the rest of the diet*, in the form of dried yeast (about 0.4 g. per *kilo unit* of food) or preferably a synthetic vitamin mixture; or the various components of the vitamin B complex may be fed as separate supplements. *Excessive* amounts of sugar in the diet of dogs will cause diarrhea, hence cereals (but not pure starch) are better sources of carbohydrate. It has been claimed that meat is an indispensable article of diet in tests on dogs. As a matter of fact, meat in the feeding of dogs is not essential if the diet is adequate with respect to protein and other nutrients.

KILO UNIT OF CASEIN FOOD*

	Amount	*Calories*	*Percentage*	
	g.			
Casein (81.9 per cent pure, 12.7 per cent N)	6.3	20.8	37.6	
Sucrose	5.84	23.4	34.9	
Lard	2.83	25.5	17.0	24.0
Butterfat	1.17	10.5	7.0	
Bone ash	0.40	..	2.3	
Salt mixture†	0.20	..	1.2	
Totals	16.74	80.2	100.0	

* This kilo unit contains 80 calories, 45 per cent of which are furnished by fat, and 0.8 g. of nitrogen.

† See footnote 91, p. 1377.

Cowgill's work shows that "*monotony of diet* is not *per se* the cause of failure of appetite: loss of the desire to eat is rather an expression either (1) of the failure of the food being fed to nourish the animal properly, or (2) of an adjustment of the dog to its energy requirement when offered more than is necessary of a food mixture that is adequate in all respects."

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per kilo unit of food contains a liberal amount of the various inorganic substances desired in the synthetic diet. Karr's salt mixture is particularly satisfactory when bone ash is used as roughage. These salt mixtures may be replaced by one of those given on p. 1375-6.

[92] Cowgill recommends the use of butterfat but lard or hydrogenated vegetable fat supplemented with codliver oil or some other source of fat-soluble vitamins may be used as the source of the bulk of the calories.

[93] Bone ash has proved very useful for this purpose.

[94] Not mentioned by Cowgill in his procedure. However, a uniform water ingestion is very essential in certain types of metabolism work (see Hawk: *Biochem Bull.*, 3, 420 (1914) and *Endocrinology and Metabolism*, Vol. 3, p. 275, New York, D. Appleton, 1924). In certain nutrition experiments the animals are given a uniform volume of water daily.

VI. ANALYSIS OF VARIANCE[95]

Table 1

ANALYSIS OF VARIANCE (ASSAY NO. 34005)

Sample: Vitamin A Concentrate
Number of groups: 4 (2 assay and 2 reference)
Assay level: 217,200 U.S.P. u./g.
Number of rats per group (N): 9 males

	S_1	S_2	U_1	U_2
Dose (mg.).	0.88 (1.5 u.)	1.47 (2.5 u.)	0.00691 ("1.5 u.")	0.01154 ("2.5 u.")
	28 Y_1	20	24	45
	31 Y_2	49	32	68
	36	65	27	38
	35	37	31	27
	30	30	22	50
	10	37	34	45
	34	36	26	58
	30	35	29	32
	24	41	13	26 Y_n
Totals...	258 (e)	350 (f)	238 (g)	389 (h)
Means...	28.7	38.9	26.4	43.2

Calculation of sums of squares:

$$C = \frac{(e + f + g \cdots n)^2}{\text{Rows} \times \text{Columns}} = \frac{(258 + 350 + 238 + 389)^2}{9 \times 4} = \frac{(1235)^2}{36} = 42367.36$$

$$\text{Columns (between doses)} = \frac{e^2 + f^2 + g^2 \cdots n^2}{\text{Rows}} - C$$

$$= \frac{258^2 + 350^2 + 238^2 + 389^2}{9} - C$$

$$= \frac{397{,}029}{9} - 42{,}367.36 = 1746.97$$

$$\begin{aligned} \text{Total} &= Y_1^2 + Y_2^2 \cdots Y_n^2 - C \\ &= 28^2 + 31^2 + 36^2 \cdots 26^2 - C \\ &= 47{,}791 - 42{,}367.36 \\ &= 5423.64 \end{aligned}$$

$$\text{Experimental error} = \text{total} - \text{columns} = 5423.64 - 1746.97 = 3676.67$$

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[95] This is included to illustrate a useful experimental design for bioassays. For references to important text-books on biometrics, see footnote 42, p. 1130.

Table 2

FACTORIAL ANALYSIS OF VARIANCE BETWEEN DOSES

Variations Due to	*Factorial Coefficients (x)*				*Divisor* $N\Sigma x^2$	*Sum of Products* ΣxYp	*Variance* $\frac{(\Sigma xYp)^2}{N\Sigma x^2}$
	S_1	S_2	U_1	U_2			
Difference between samples	−1	−1	+1	+1	36*	+19†	10.03‡ = D^2 3.17 = D
Slope of dose response curve	−1	+1	−1	+1	36	+243	1640.25 = B^2 40.50 = B
Departure from parallelism	+1	−1	−1	+1	36	+59	96.69
Total (Yp).	258 (e)	350 (f)	238 (g)	389 (h)	..	..	..

Note: D and B take the signs of the Sums of Products from which they are derived.

* $9\ ((-1)^2 + (-1)^2 + (+1)^2 + (+1)^2) = 36.$

† $(-1)258 + (-1)350 + (+1)238 + (+1)389 = 19.$

‡ $\frac{(19)^2}{36} = 10.03.$

Table 3

ANALYSIS OF VARIANCE

Variations Due to	*Degrees of Freedom (DF)*	*Sum of Squares (SS)*	*Mean Square (SS ÷ DF = MS)*	*Variance Ratio (MS ÷ s^2 = F)*	*Significant F**
Difference between samples..	1	10.03	10.03	0.09	4.15
Slope of dose-response curve	1	1640.25	1640.25	14.28	4.15
Departure from parallelism..	1	96.69	96.69	0.84	4.15
Experimental error..........	32	3676.67	114.90 (s^2)	1	..
Total....................	35	5423.64	..	..	..

$$s = \sqrt{114.90} = 10.72$$

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* From Snedecor: *Statistical Methods Applied to Experiments in Agriculture and Biology*, 4th ed. Ames, Iowa, Collegiate Press, 1946, p. 224. See also footnote 42, p. 1130.

Table 4

CALCULATION OF ESTIMATE OF POTENCY

$$M = \log \frac{\text{potency of } U}{\text{potency of } S} = \frac{KID}{B}$$

$K = 1$ (for two-dose assays; for three-dose assays $K = 1.635$)

$$I = \log \frac{\text{large dose}}{\text{small dose}} = \log 1.667 = 0.2219$$

$$\left.\begin{array}{l} D = 3.17 \\ B = 40.50 \end{array}\right\} \text{from factorial analysis (Table 2, on p. 1379)}$$

$$M = \frac{1 \times 0.2219 \times 3.17}{40.50} = 0.0174$$

Log estimated potency of sample (EP) = log of assumed potency of sample (AP) + M

$$\begin{aligned} \text{Log } (AP) = \log 217{,}200 &= 5.3369 \\ M &= \underline{0.0174} \\ \log (EP) &= 5.3543 \end{aligned}$$

antilog 5.3543 = 226,100 = Estimated potency of sample in vitamin A units per gram

Table 5

CALCULATION OF ERROR OF ESTIMATE

$$S_M = \text{log standard error} = \frac{sKI\sqrt{B^2 + D^2}}{B^2}$$

where

s = standard deviation of assay = 10.72

$K = 1$

$I = 0.2219$

$$\left.\begin{array}{l} B^2 = 1640.25 \\ D^2 = 10.03 \end{array}\right\} \text{from factorial analysis (Table 2, above)}$$

$$S_M = \frac{10.72 \times 1 \times 0.2219 \times \sqrt{1650.28}}{1640.25} = 0.0589$$

$$\begin{aligned} \log (EP) \pm S_M &= \log \text{ (limits of potency)} \\ 5.3543 + 0.0589 &= 5.4132 \\ 5.3543 - 0.0589 &= 5.2954 \\ \text{antilog } 5.4132 &= 259{,}000 \\ \text{antilog } 5.2954 &= 197{,}400 \\ \text{Difference} &= 61{,}600 \\ \tfrac{1}{2} \text{ difference} &= 30{,}800 \end{aligned}$$

Estimated potency and standard error = 226,100 ± 30,800 units of vitamin A per gram.

Standard error as per cent of estimate = 13.6 per cent

VII. FOUR-PLACE TABLE OF LOGARITHMS

Natural Numbers	0	1	2	3	4	5	6	7	8	9	*Proportional Parts*								
											1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	8	12	17	21	25	29	33	37
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5052	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7

FOUR-PLACE TABLE OF LOGARITHMS—(*Continued*)

Natural Numbers	0	1	2	3	4	5	6	7	8	9	*Proportional Parts*								
											1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4

VIII. TABLE OF INTERNATIONAL ATOMIC WEIGHTS, 1953

Element	*Symbol*	*At. No.*	*At. Wt.**	*Element*	*Symbol*	*At. No.*	*At. Wt.**
Actinium	Ac	89	227	Mercury	Hg	80	200.61
Aluminum	Al	13	26.98	Molybdenum	Mo	42	95.95
Americium	Am	95	[243]	Neodymium	Nd	60	144.27
Antimony	Sb	51	121.76	Neon	Ne	10	20.183
Argon	A	18	39.944	Neptunium	Np	93	[237]
Astatine	At	85	[210]	Nickel	Ni	28	58.69
Arsenic	As	33	74.91	Niobium	Nb	41	92.91
Barium	Ba	56	137.36	Nitrogen	N	7	14.008
Berkelium	Bk	97	[245]	Osmium	Os	76	190.2
Beryllium	Be	4	9.013	Oxygen	O	8	16
Bismuth	Bi	83	209.00	Palladium	Pd	46	106.7
Boron	B	5	10.82	Phosphorus	P	15	30.975
Bromine	Br	35	79.916	Platinum	Pt	78	195.23
Cadmium	Cd	48	112.41	Plutonium	Pu	94	[242]
Calcium	Ca	20	40.08	Polonium	Po	84	210
Californium	Cf	98	[246]	Potassium	K	19	39.100
Carbon	C	6	12.011	Praseodymium	Pr	59	140.92
Cerium	Ce	58	140.13	Promethium	Pm	61	[145]
Cesium	Cs	55	132.91	Protactinium	Pa	91	231
Chlorine	Cl	17	35.457	Radium	Ra	88	226.05
Chromium	Cr	24	52.01	Radon	Rn	86	222
Cobalt	Co	27	58.94	Rhenium	Re	75	186.31
Columbium				Rhodium	Rh	45	102.91
See Niobium				Rubidium	Rb	37	85.48
Copper	Cu	29	63.54	Ruthenium	Ru	44	101.1
Curium	Cm	96	[243]	Samarium	Sm	62	150.43
Dysprosium	Dy	66	162.46	Scandium	Sc	21	44.96
Erbium	Er	68	167.2	Selenium	Se	34	78.96
Europium	Eu	63	152.0	Silicon	Si	14	28.06
Fluorine	F	9	19.00	Silver	Ag	47	107.880
Francium	Fr	87	[223]	Sodium	Na	11	22.991
Gadolinium	Gd	64	156.9	Strontium	Sr	38	87.63
Gallium	Ga	31	69.72	Sulfur	S	16	32.066†
Germanium	Ge	32	72.60	Tantalum	Ta	73	180.95
Gold	Au	79	197.0	Technetium	Tc	43	[99]
Hafnium	Hf	72	178.6	Tellurium	Te	52	127.61
Helium	He	2	4.003	Terbium	Tb	65	158.93
Holmium	Ho	67	164.94	Thallium	Tl	81	204.39
Hydrogen	H	1	1.0080	Thorium	Th	90	232.05
Indium	In	49	114.76	Thulium	Tm	69	168.94
Iodine	I	53	126.91	Tin	Sn	50	118.70
Iridium	Ir	77	192.2	Titanium	Ti	22	47.90
Iron	Fe	26	55.85	Tungsten	W	74	183.92
Krypton	Kr	36	83.80	Uranium	U	92	238.07
Lanthanum	La	57	138.92	Vanadium	V	23	50.95
Lead	Pb	82	207.21	Xenon	Xe	54	131.3
Lithium	Li	3	6.940	Ytterbium	Yb	70	173.04
Lutecium	Lu	71	174.99	Yttrium	Y	39	88.92
Magnesium	Mg	12	24.32	Zinc	Zn	30	65.38
Manganese	Mn	25	54.94	Zirconium	Zr	40	91.22

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* A value given in brackets denotes the mass number of the isotope of longest known half-life.

† Because of natural variations in the relative abundances of the isotopes of sulfur, the atomic weight of this element has a range of ±0.003.

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